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On the distribution of assimilated iron compounds ... in animal and vegetable cells

A. B. Macallum

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On the Distribution of Assimilated Iron Compounds, other than Hæmoglobin and Hæmatins, in Animal and Vegetable Cells.

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With Plates 10—12.

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I. Preliminary Remarks.

In 1891, in a communication to the Royal Society,[1] I described a method by which the presence of iron in the chromatin of

[1] "On the Demonstration of the Presence of Iron in Chromatin by Microchemical Methods," 'Proceedings Roy. Soc.,' vol. l, p. 277.

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animal and vegetable cells may be demonstrated micro-chemically, and I referred to the results then obtained with it as indicating, apparently, that iron is always a constituent element of this substance. The interest which the subject had for me led me to continue the investigation with improved methods of research, and I am now consequently in a position to describe a much more extensive series of observations in support of the generalisation, then somewhat tentatively advanced, that iron is a constant constituent of the nuclein substance proper.

From the commencement of the investigation I have been fully aware of its difficulties, and I can, therefore, readily understand that view of the subject which led Gilson to remark that the solution of the question concerned is one "that seems to require more than a single man's activity."[1] The difficulties encountered in the application of the micro-chemical method are, however, very much less formidable than those met with in the employment of the older methods. I have pointed out, in my first paper on this subject, how impossible it is to be certain that the iron revealed by macro-chemical methods in isolated quantities of nuclein is not present through absorption from some other source, but due to a combination obtaining in unisolated living chromatin, and I have indicated that the only way in which the question could be settled definitely is by the employment of micro-chemical methods. I have shown in the succeeding pages of this paper that the acid alcohol upon which Bunge relied to extract the iron of inorganic and albuminate compounds from egg-yolk and other nuclein-holding substances, and leave intact the organic (nucleinic) iron, does not perform this function at all when the substance treated with it is in mass, while it removes the iron of all three classes of compounds from thin sections of tissues, if the time allowed for its action be prolonged. We have, consequently, in a macro-chemical investigation, no means whatever of distinguishing between organic iron on the one hand and the iron of inorganic and albuminate combinations on the other, and we are therefore

[1] "On the Affinity of Nuclein for Iron and other Substances," 'Report British Association for the Advancement of Science,' 1892, p. 778.

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forced, more than ever, to depend on micro-chemical methods to determine the relations of assimilated iron to the cell. Objections may be urged against these methods also, based chiefly on the facts that iron, free or combined, contaminates everything, so to speak, and that what is shown to occur in dead chromatin may not be present in the living compound; but these objections at once lose their force when the methods are applied with all due care accompanied by such control experiments as the conditions may suggest.

I have in my former communication made reference to the investigations of Bunge and Zaleski upon iron-holding nucleins. Since 1891 four other investigators have published observations on the occurrence of iron in organic compounds.

Molisch[1] endeavoured to determine the relations of iron in the vegetable cell by means of concentrated aqueous solutions of potash. He found that when vegetable tissues were immersed in this reagent for a day or longer, they gave a reaction for iron not at all obtainable in the fresh tissues, and he explained the result as due to the removal of the iron from a firmly combined ("maskirt") condition to that in which it is readily detectable by ordinary reagents. The firmly combined iron, as shown by this method, was sometimes in the cell wall, sometimes in the cell contents, and sometimes again in both. His results do not call for a fuller description than this, since in a later publication[2] he has stated that his solutions of potash were not free from iron, and he has consequently withdrawn all the conclusions which he previously based on the results obtained with this reagent.

Petit,[3] in investigating the occurrence of iron in barley, employed Bunge's method to separate the inorganic and albuminate from the organic iron, using for that purpose a 1 per cent. solution of hydrochloric acid in absolute alcohol.

[1] 'Die Pflanze in ihren Beziehungen zum Eisen,' Jena, 1892.

[2] "Bemerkung über den Nachweis von maskirterm Eisen," 'Berichte der deutschen bot. Gesell.,' vol. xi, 1893, p. 73.

[3] "Distribution et l'état du fer dans l'orge," 'Comptes Rendus,' vol. cxv, p. 246, 1892.

The dried and finely pulverised barley was put, with the acid alcohol, in a Soxhlet extraction apparatus and heat was applied for six hours, during which time the reagent was renewed once, but the second liquid extracted no iron. The result was the same when the strength of the acid in the solution was 2·5 per cent. From his experiments he concludes that nearly all the iron is combined with nuclein (à l'état de nucléine) and exclusively contained in the tegmen and embryo of the barley grain. In a second publication[1] he describes the separation of an iron-holding nuclein from the malt-combs (touraillons) of barley, free from sulphur and in which the iron amounted to 0·195 per cent. The separation was made by extracting the pulverised matter with a 1 per cent. solution of potash at 60° C. for some minutes, and filtering off under pressure the brown liquid, which was then neutralised with dilute hydrochloric acid. The precipitate formed was washed by decantation with water, then with alcohol and ether, and finally dried over sulphuric acid.

Gilson[2] found iron in the nucleinic elements, not only when ammonium sulphide, according to my method of using it, was employed, but also after treatment with other reagents and in nuclei which, without such treatment, gave no reaction for iron with the ordinary methods of demonstration. He specially mentions sulphuric acid and sulphurous anhydride as giving the best results, although others, among which he includes saline solutions, produce the same effects. He is, however, inclined to regard the iron demonstrated in the nuclein as due to a combination which is formed only after death, and similar to that which dead nuclein effects with many other substances, especially colouring matters. He showed that dead nuclein has a very strong affinity for iron compounds, the nuclei of freshly extracted cells absorbing from a 0·05 per cent. solution of ferrous sulphate more iron than could be demonstrated in them when simply treated with sulphuric acid; and he maintains it is extremely difficult to ascertain whether nuclein in a living

[1] "Sur une nucléine végétale," 'Comptes Rendus,' vol. cxvi, p. 995. 1893.

[2] Loc. cit.

condition contains iron, or contains it only after death, deriving it by absorption from the blood or other surrounding fluids, or even out of the reagents themselves, if these are not absolutely free from iron. In his remarks upon my methods he states that Bunge's fluid, upon which I relied to extract the iron of inorganic and albuminate combinations from sections of tissues, does not take away the iron artificially combined with dead nuclein even after six days.

Hammarsten[1] has isolated from the pancreas of the ox an iron-holding nucleo-proteid containing 4·48 per cent. of phosphorus.

II. Methods of Study.

In my first communication on the method of demonstrating micro-chemically the occurence of "masked" iron, the reagent whose use I described was called, in a general way, ammonium sulphide. This is a term that is properly applicable to the diammonium compound represented by the formula $(NH_4)_2S$, but it is sometimes given to solutions which contain ammonium hydrogen sulphide (NH_4HS), or polysulphides of ammonium, or to mixtures of diammonium sulphide and ammonium hydrogen sulphide. At the time I was unable to determine which of the two latter is the most effective as a reagent in liberating the iron from the chromatin, since either, when recently prepared, gave, with cellular elements from the same piece of tissue, reactions in which differences in intensity were not noticeable, and, while uncertain upon this point, I felt justified in adopting the generic term "ammonium sulphide" to designate a reagent which might be held to indicate either of the two compounds.

About two years ago I gave further attention to the question whether one form of the reagent is more efficient than the other in this respect, and the results of a series of experiments made since then have led me to the conclusion that ammonium

[1] "Zur Kenntniss der Nucleo-proteïde," 'Zeit. für Physiol. Chemie,' vol. xix, 1894, p. 19.

hydrogen sulphide is more active than the diammonium salt, and that none of the polysulphides of ammonium have any action whatever on iron in its "masked" form. These experiments have been controlled by others made with these reagents upon solutions of potassium ferrocyanide.[1] Ammonium sulphide, when mixed with a solution of the latter salt and the mixture kept at a temperature of 30—50° C. for one or more days, will liberate the iron from its combination and precipitate it as sulphide, the amount so liberated depending on the strengths of the solutions forming the mixture, on the temperature and on the time during which the reaction is allowed to go on. A lower temperature will suffice when the time is prolonged. By paying due attention to all the conditions, it is possible to liberate, as sulphide, all the iron of such solutions. In this ammonium hydrogen sulphide is more active than diammonium sulphide, the amount of the sulphide formed being a measure of the activity of either reagent.[2] These experiments have, in all cases, given results which correspond with those obtained with the two sulphides upon the chromatin of isolated cells, but it was not possible in the latter case to estimate the effects as definitely. I found that of two slide preparations of isolated cells, one made with ammonium hydrogen sulphide, the other with diammonium sulphide, the former as a rule gave the

[1] I have not found any reference to the action of ammonium sulphide on solutions of ferrocyanides in the literature of chemistry, although, on the presumption that some such reference exists, I made diligent search for it.

[2] The results of one experiment upon this point may be mentioned. The glass-stoppered cylinder *a* contained 10 c.c. of a 10 per cent. solution of potassic ferrocyanide and 10 c.c. of ammonium hydrogen sulphide made from an ammonia solution of 0·96 sp. gr., while to a similar cylinder *b*, with like quantities of the same solutions, 10 c.c. of dilute ammonia were added. At the end of twenty-four hours' stay in a warm oven with a temperature of 40° C., the precipitates were filtered off with iron-free filters, washed with water containing hydrogen sulphide in solution, dissolved in dilute sulphuric acid solutions, and, after care had been taken to reduce all the iron to the ferrous condition, the amount of the metal in each case was estimated by titration with a standardised permanganate solution. Results: the precipitate in *a* contained 0·0113 grm. iron, while the iron of the precipitate in *b* amounted to 0·0025 grm.

maximum reaction in about ten days, while the latter manifested a reaction of moderate intensity at the end of that time, which, with a longer stay in the warm oven, did not become more marked. In the case of vegetable cells the reactions were more quickly obtained and the differences in intensity greater. This is illustrated in figs. 14, 15, and 16, representing preparations of cells of the ovary of Erythronium americanum, in which the reagents used had been made from dilute solutions of ammonia (of 0·96 sp. gr.). Fig. 15 indicates the depth of the reaction with ammonium hydrogen sulphide at the end of twenty-four hours, the intensity attaining in another cell in ninety hours the degree represented in fig. 16, while in fig. 14 is shown how far the reaction had progressed with diammonium sulphide in forty-eight hours. In the latter case the reaction did not become more marked even on the eighth day. Similar results were obtained in all the experiments of this character, demonstrating that ammonium hydrogen sulphide is more effective in liberating iron from organic combinations than is the diammonium compound.

In the earlier stages of the investigation the reagent was made from strong solutions of ammonia of sp. gr. 0·88; but when thus prepared it deteriorates rapidly and becomes yellow from the formation of polysulphides. Spoiled or unsuccessful preparations were consequently frequently obtained. Sometimes, also, difficulties were experienced in determining whether, in the preparation of the reagent, the saturation of the strong ammonia with sulphuretted hydrogen was complete. For this reason, and also because dilute solutions of ammonium hydrogen sulphide are less unpleasant in every way, I began to use the latter, and found that it gives results not less decided than those obtained with the stronger solutions. The dilute solutions offer other advantages, for when made from pure ammonia of 0·96 sp. gr., they retain their potency for three weeks or longer, especially if kept in a bottle with a well-fitted glass stopper, and in a cool place. The smaller the amount of air in the bottle and the less frequently the stopper is removed, the longer does the reagent retain its strength.

During the last two years the dilute reagent has, in consequence of these facts, been exclusively employed.

The glycerine used was chemically pure.[1] It gave the best results when diluted with an equal volume of distilled water. In making the preparations, the cellular elements were teased out on the slide in a drop of the dilute glycerine, and over this, after thorough admixture with two drops of the dilute solution of ammonium hydrogen sulphide, a cover-glass of 16—22 mm. square was placed. The teasing-out process was done in each case with a clean pair of goose-quill points. Every care was taken to prevent the occurrence of impurities in the preparations. The excess of the glycerine and sulphide mixture is at first uncovered, but if the slide be put in a warm oven with a temperature of 60° C., the mixture rapidly concentrates and in a few minutes is wholly under the cover-glass. When the solution of ammonium hydrogen sulphide is deteriorated, a deposit of sulphur forms at the edges of the cover-glass and the mixture under the latter becomes yellow through the production of polysulphides of ammonium. Such preparations never yield anything of value. On the other hand, when the fluid under the cover-glass remains colourless and free sulphur does not form at the margins, the preparation, if kept at a temperature of 55—60° C. for a period of from two to fifteen days, is almost always successful. Sometimes at the end of one, two, or three days the mixture is further concentrated and has receded from one edge of the cover-glass. This is remedied

[1] Molisch ('Die Pflanze in ihren Beziehungen zum Eisen,' p. 107) states that the glycerine of commerce—even the purest—contains traces of iron. I have not found this to be the case with Price's glycerine, quantities of which, when mixed with ammonium hydrogen sulphide or diammonium sulphide, gave not the slightest reaction or precipitate, even after two weeks, and whenever portions of the stock supply used were evaporated at a low heat in a platinum dish no appreciable residue was left, and not a trace of iron or lead was detected. I found that in some samples of glycerine of other manufacture the sulphide gave no immediate reaction, but at the end of a week, or later, a small precipitate, composed partly of sulphide of iron, was at the bottom of the test-tube. A similar precipitate was obtained in portions of the stock supply of Price's glycerine only when traces of an iron salt were added.

by placing at the dry side of the cover-glass a drop of a mixture of one part of dilute glycerine and two of ammonium hydrogen sulphide, the drop so placed running under the cover, after which the preparation is replaced in the warm oven and in the end usually proves successful. I have found that when the isolated cellular elements are not very numerous and uniformly distributed under the cover-glass, evaporation rarely goes so far as to render a resort to this remedy necessary; but when the tissues are only partially teased, and fragments tilt or elevate the cover-glass, the mixture concentrates, the preparation dries at one side, and the sulphide is largely converted into polysulphide.

The solutions of ammonia used in the preparation of the reagents were chemically pure, and in this respect, as well as in the cleanliness of the slides and covers, I paid due regard to the suspicion that there possibly exists a ferrous sulphc-hydrate (FeS_2H_2), soluble to a certain extent in solutions of ammonium hydrogen sulphide, the presence of which in the glycerine and sulphide mixture of my preparations might, through its diffusion into the nuclei and precipitation therein as ferrous sulphide (FeS), give confusing results. That no such compound existed in my reagents was shown repeatedly by allowing mixtures of the sulphide and glycerine to stand for weeks, when all the ammonium hydrogen sulphide was converted into the diammonium salt, or into polysulphides of ammonium, in the presence of which it would appear that the supposed existence of ferrous sulpho-hydrate is impossible. In these experiments no iron was found, nor did the mixtures in the end lose any of their transparency,—a result which tells against the possibility of any such iron compound existing in the mixtures employed upon teased-out cells. The cover-glasses and slides were cleaned in solutions of hydrochloric acid to remove any adherent compounds of iron, and afterwards passed through distilled water and alcohol. The bottles in which the solutions of ammonium hydrogen sulphide were kept were also, first of all, cleansed in the same way.

Nothing was gained by making "stock" mixtures, in the

proper proportions, of glycerine and ammonium hydrogen sulphide, for in such the reagent is more rapidly converted into the non-active form than when it is kept separate. Apparently also in "stock" mixtures the polysulphides are very rapidly formed, the fluids becoming deep yellow in twenty-four hours or less, although the sulphide used may be nearly colourless. In summer the change of colour is rapid. That it is due in part at least to the formation of polysulphides, appears to follow from the fact that drops of the mixture, when allowed to remain uncovered on the slide for a few minutes, quickly become milky in appearance from the precipitation of free sulphur. The mixtures retain a part of their strength during the first two or three days, after which they become useless.

The tissues which were teased out for treatment were always hardened in alcohol wholly free from iron in solution. Latterly I have employed for this purpose redistilled methylated spirit. I have not used in this connection material fixed with any of the mineral hardening reagents, since the latter frequently contain iron, the presence of which in dying cells and tissues might be held to contribute, under the influence of the hardening reagent, to the formation of firm organic compounds of iron. Some mineral reagents, moreover—as, for example, corrosive sublimate and osmic acid—are difficult to remove from the tissues upon which they have been allowed to act, and their presence in preparations treated with ammonium sulphide, which forms sulphides with these metals, gives appearances obscuring, in a greater or less degree, the occurrence of iron compounds.

To facilitate the teasing-out I frequently used sections made with a clean steel knife[1] covered with absolute alcohol, the cells of such sections readily separating, and yielding sometimes a number of free nuclei. In order to determine whether iron in an inorganic or albuminate form is present, and to what

[1] In my earlier paper (loc. cit.) I pointed out that the knife so used gives no iron to the preparation. All my observations for the last two and a half years have in no way called in question the correctness of this contention.

extent, it was my practice to allow the section to lie in the glycerine and sulphide mixture for a few minutes before teasing it out, the iron of these forms of combination giving an immediate reaction on the penetration of the reagent. The removal of all iron of this description is necessary, since its presence may give confusing results in teased-out cells. For this purpose I have used Bunge's fluid, in which the sections were kept for about an hour with the reagent at a temperature of 55° C., the subsequent treatment with alcohol and ammonium hydrogen sulphide in all cases showing that the inorganic and albuminate iron had been thereby removed.[1] Sections so treated were teased out and mounted in the glycerine and sulphide mixture in the usual way.

The disadvantages connected with the use of ammonium hydrogen sulphide to demonstrate the presence of "masked" iron are that it effects, in the animal cell at least, structural changes, that it is not successful on large nuclei or on nuclei of large cells, and that it requires a great expenditure of time. In regard to the structural changes it is obvious that, however well hardened or well fixed cellular elements may be through the action of alcohol, ammonium hydrogen sulphide or diammonium sulphide must, when heat is applied, sometimes alter, to a greater or less degree, the structure of the cell, and especially of its nucleus. This is quite evident when we compare such preparations with others in which the "masked" iron has been liberated by the use of sulphuric acid alcohol, and subsequently treated with the sulphide. Figs. 23 and 24 illustrate the differences obtained with the two methods, the former representing liver-cells of Necturus lateralis treated for ten days at 55° C. with the glycerine and sulphide mixture, while the latter was drawn from a section of the same material after it had been acted on by sulphuric acid alcohol for seven hours at 35° C., and then with the glycerine and sulphide mixture. The first difference to be noted between the

[1] In regard to the capacity of Bunge's fluid for extracting iron of all forms of combination, see the description of the properties of hydrochloric acid alcohol as given below.

preparations represented is that of the iron reaction illustrated. This is partly due to the fact that in one preparation the ammonium hydrogen sulphide has not liberated all the iron of the chromatin, but partly also to the fact that the reagent has caused the delicate chromatin elements to become swollen, thereby rendering the iron reaction more diffuse and less marked. The effect on the cytoplasm is not less striking. It is, however, chiefly with concentrated solutions of ammonium hydrogen sulphide that preparations of animal nuclei exhibit this phenomenon. Solutions of the reagent made from ammonia of 0·96 specific gravity do not as readily produce this change, and in many cases none at all may be shown. When the reagent is fresh the reaction is quickly obtained, sometimes in two or three days, and then no swelling of the nuclear network occurs; but when it is not fresh, or when it gives an odour of ammonia, the reaction is slowly obtained, and the prolonged application necessary in order to bring out this result, aided perhaps by the ammonia, causes a swelling of the chromatic elements.

The slowness with which the reaction comes out is not wholly a disadvantage, for by this means one may determine whether the iron demonstrated is derived from other than inorganic or albuminate compounds. With the exception of hæmoglobin, hæmatin, and the compound found in yolk-spherules, the organic combinations in which the iron is "masked" are affected very slowly by ammonium hydrogen sulphide, and only when heat is applied; whereas the reaction comes out at once, or after a few minutes at the longest, and without heat, in the case of inorganic and albuminate compounds. The distinction between these and the "masked" compounds is, therefore, very marked. In one of the exceptions mentioned the distinction is not so clear, for when ammonium hydrogen sulphide is added to the fresh yolk of hen's egg it gives a greenish reaction at once, but when the yolk is hardened with alcohol or with heat the reagent gives this result only after several days' application at 50—60° C. On the other hand, the yolk-spherules in Amphibia (Necturus

and Amblystoma), whether hardened or fresh, yield the reaction in a few minutes. Such compounds are of too limited a range of distribution to affect the value of the reagent in making a distinction between the iron compounds. On hæmoglobin and myo-hæmatin (myo-hæmoglobin) the reagent has not the slightest action. I have kept mixtures of the reagent with solutions of hæmoglobin and myo-hæmoglobin for more than a year at a temperature of 55° C., and in no case have I found that iron was liberated from these compounds as sulphide. I have, moreover, mounted in the glycerine and sulphide mixture on the slide finely powdered hæmoglobin which had been coagulated in alcohol, and applied heat to the preparation for weeks without once obtaining the iron in an inorganic form. When, therefore, in preparations of animal tissues which have been hardened in alcohol one obtains with the glycerine and sulphide method after a time an iron reaction, it may reasonably be concluded that the iron so demonstrated is not derived from hæmoglobin in the tissues. One may not, however, exclude hæmatin as a possible source of iron, for although hæmoglobin in all forms will not yield its iron to ammonium hydrogen sulphide, the latter readily liberates the iron of hæmatin, and from a solution of hæmatin in ammoniated alcohol or in dilute ammonia, into which hydrogen sulphide has been passed, part of the iron at ordinary temperatures, but the whole at 50° C., is precipitated as ferrous sulphide, in a few days.[1] Even in a solution of hæmatin in ammoniated alcohol, if kept for several days at the temperature of the room,

[1] The compound formed from the hæmatin in this process of liberating the iron is neither hæmatoporphyrin nor bilirubin. With yellow nitric acid it gives a play of colours in which violet, faint red, and yellow successively appear, the mixture finally becoming colourless, and it yields an absorption spectrum like that of bilirubin. It is insoluble in ether, and soluble in chloroform and hot alcohol. The other properties of this compound are now under investigation. It has one special claim to interest in that it is formed from hæmatin by a method very much less drastic in its effects than those in which strong sulphuric acid or bromine in glacial acetic acid is used to form hæmatoporphyrin or bilirubin (Nencki and Sieber, 'Monatsh. für Chemie,' vol. ix, p. 115, 1888).

a part of the iron of the hæmatin is precipitated as a greyish-white hydroxide, which, if filtered off, gives at once with ammonium sulphide the greenish-black sulphide reaction. Very weak solutions of hydrochloric and other acids effect the removal of the iron, and if solutions of hæmatin in alcohol are kept for a week or more in contact with solutions of various salts (potassium chlorate and sulphate and sodium chloride and phosphate), decomposition of the hæmatin results, and iron is liberated as an inorganic compound. In all these respects hæmatin behaves like the ferrocyanides, while it differs markedly from hæmoglobin in the same points.

Experiments show, however, how little, if any, of the iron demonstrated in animal cells is derived from hæmatin. Sections of the liver and other organs of Vertebrates, as well as of vegetable tissues, were placed in alcoholic solutions of hæmatin for twenty-four hours, then washed in alcohol for a few minutes, and kept in a quantity of the glycerine and sulphide mixture at a temperature of 35° C. for twenty-four hours. At the end of the latter interval all the sections were blackened, and under the microscope the nuclei were dark green from the ferrous sulphide liberated from the hæmatin absorbed by the chromatin. In order to get this result the sections do not require to be teased out at all. The rapidity with which such a strong reaction is obtained indicates that in ordinary teased-out cells mounted on the slide in the glycerine and sulphide mixture, the deep reactions obtained after several days or after a week are due to a decomposition, not of hæmatin, but of some other compound or compounds.

Ammonium hydrogen sulphide, then, may be regarded as a reagent of very great value in the investigation of "masked" compounds of iron, and it must constitute a final test for this purpose, whenever the accuracy of the other reagents, used also for determining the distribution of assimilated iron compounds in cells, is called in question.

In June, 1891, Mr. R. R. Bensley, while carrying on under my direction a research on the distribution of iron in the ovary of Erythronium americanum, as demonstrated by

the employment of ammonium sulphide, succeeded in obtaining some interesting results which necessitated control experiments based on the removal of all traces of inorganic compounds of iron from the tissues under investigation. For this purpose Bunge's fluid was used, and it was thought that hardened specimens of the ovary, when subjected to its action for a time, would not give, on the addition of ammonium sulphide, any immediate reaction for iron, and that further treatment with the latter reagent in a warm oven for several days would show the presence of iron in the nuclei of their cells, and possibly also in their cytoplasm. Much to our surprise, however, the treatment of the ovary of Erythronium with a quantity of Bunge's fluid for two weeks at 20° C., and the subsequent application of ammonium sulphide, resulted in the production of a marked reaction for iron, which under the microscope was found confined to the nuclei. I was at first inclined to believe that the iron so shown was due to diffusion into the nuclei of that present in an inorganic form in the tissues, and this would appear to be Gilson's view; but repeated experiments have demonstrated the incorrectness of this explanation, and that Bunge's fluid liberates the iron of organic compounds.[1] Experiments were also made on animal tissues and similar results were obtained. The liberation of the iron is to be attributed to the hydrochloric acid, the only active part of the reagent. This conclusion suggested a number of experiments, all based on the principle that whatever proper-

[1] Gilson's statement is difficult to interpret. He does not say whether he applied the reagent to sections of tissues or to the latter in mass, and at what temperature it was allowed to act. He appears to regard the iron absorbed by dead nuclein as combined with the latter, and he remarks, in reference to my statement that Bunge's fluid removes all inorganic and albuminate iron from sections after treatment with it for ten hours: "but I have observed that Bunge's liquid does not take away the iron artificially combined with dead nuclein after six days." I can explain his statement only on the supposition that he used the reagent on the tissues in mass, and that he thereby obtained the same results that I did under similar circumstances; in other words, the iron "artificially combined with dead nuclein" was in reality iron liberated by Bunge's fluid from its masked condition in the chromatin and retained by the latter.

ties hydrochloric acid may have in this respect are possessed, in a greater or less degree, by other mineral acids, whether in dilute aqueous solutions or in alcohol, and the results were of such a character as to induce me to employ these reagents on all species of cells in which the distribution of iron had been determined with ammonium hydrogen sulphide.

The more serviceable of these were found to be sulphuric acid and nitric acid dissolved in alcohol of 95 per cent. strength. The former was prepared by adding four volumes of the strong acid to one hundred of alcohol, while the latter contained three volumes of the acid (of 1·4 sp. gr.) in one hundred of alcohol.

The chemicals used in the preparation of these reagents were free from traces of iron, and care was taken to have all bottles and vessels used to hold them also free from adherent iron compounds. It was, of course, impossible to provide against the iron in the glass, but I am not certain that the reagents derived any from this source, even in infinitesimal quantities. The alcohol used contained not a trace of iron. During the last eighteen months re-distilled methylated spirit was found to be in every way as serviceable as the pure ethyl alcohol used earlier in the investigation.

The alcohol of these reagents largely prevents the occurrence of digestive changes which the acids effect when, in aqueous solutions, they are allowed to act on tissues for several days, and especially at a slightly elevated temperature. Another important function of the alcohol is to prevent a too rapid extraction of the liberated iron, and thereby also its diffusion from one part of the tissue into another, from nucleus to cell, or from cell to nucleus. Acid alcohols dissolve iron salts more readily than does alcohol alone, but less so than aqueous solutions of the acids. For example, ferrous sulphate is insoluble in absolute alcohol and in strong methylated spirit, but it is soluble in these when they contain a small quantity of sulphuric acid,—not, however, in any way as much so as in distilled water, or in dilute aqueous solutions of sulphuric acid. The smaller the proportion of the acid in the alcohol

the less readily does it dissolve the iron salts, and when used upon tissues acid alcohols have a smaller capacity for extracting the iron salts the longer the reagents are allowed to act, for the liberation of the iron from its "masked" condition entails the neutralisation of the acid, a very gradual process. As a result of this neutralisation the iron salts become less soluble and would pass back into the tissues, but the danger of this happening is minimised or altogether prevented by the property which the chromatin has of retaining the iron that is set free in itself by the acid alcohol. This is shown specially in the case of nitric acid alcohol, for when sections of vegetable tissues are allowed to lie for two weeks in a large quantity of the reagent, the nuclei at the end of that time give as intense a reaction for iron as they do at the end of two days. The result is due to the fact that the tenacity with which the chromatin holds the iron liberated in it counteracts the extractive capacity of the reagent.

The results of the action of nitric and sulphuric acid alcohols differ from those obtained with Bunge's fluid[1] in one important respect. The two former, whether they are used upon the tissues in mass or on sections of the same, leave the iron, on the whole, in the parts in which it is liberated; but when sections of tissues are treated with hydrochloric acid alcohol, the iron is extracted as quickly as it is liberated, and consequently such preparations on treatment with ammonium sulphide give a feeble reaction for iron or none at all. This is most distinctly seen when the temperature is raised, and if the reagent is allowed to act for two or three days under these conditions, no iron, organic or inorganic, is left in the preparations. When the tissues are in mass, on the other hand, the quantity of acid that penetrates the preparations is largely neutralised and extraction takes place very slowly, with the result that teased-out portions of such tissues give a marked reaction for iron, limited, as in preparations obtained with

[1] Bunge's fluid, or hydrochloric acid alcohol, consists of ninety volumes of alcohol of 95 per cent. strength, and ten volumes of a 25 per cent. solution of hydrochloric acid.

the other acid alcohols, to the parts in which treatment with the glycerine and sulphide mixture demonstrates its occurrence.

In describing the properties of hydrochloric acid alcohol, Bunge expressly states that while it extracts inorganic iron it does not remove the iron from the nuclein (hæmatogen) of egg-yolk.[1] This is not quite correct, for when hard-boiled yolk is treated with ammonium sulphide it gives only a feeble reaction for iron, even when kept for twenty-four hours at an elevated temperature; but when it has been acted on by a quantity of Bunge's fluid for a day at 30—35° C., the application of ammonium sulphide, after all traces of the acid have been removed with alcohol, gives an immediate and marked reaction for iron. The iron under such conditions must be in the form of chloride, and as an inorganic compound it should be extracted by the reagent, if Bunge's views concerning the properties of the latter be correct, but this happens only when the quantity of the yolk so treated is very small, and then the whole of the iron is removed in a few days, this fact demonstrating clearly that the reagent in its action makes no distinction between inorganic and organic iron. The latter is in its liberation from the "masked" condition converted into the inorganic form, and it depends on the quantity of yolk used whether or not the extraction may keep pace with this conversion. If the quantity is large, the liberation of the iron from its organic combination entails a diminution of the acidity of the reagent, and at length the extraction of the liberated iron ceases. It commences again only when a fresh quantity of the reagent is substituted for the exhausted fluid.

The results of its action upon the iron-containing nucleo-albumin of yolk are therefore practically similar to those which it gives when applied to animal and vegetable tissues.

The fact that a considerable diminution of the acidity of hydrochloric acid alcohol allows the liberated iron to be retained

[1] "Ueber die Assimilation des Eisens," 'Zeit für Physiol. Chemie,' vol. ix, 1885, p. 49.

in its original position in the cell has led me to try the effects of solutions in which the strength of the acid was less than 1 per cent.,[1] and they have been found, when used upon thin sections of tissues, to give very successful preparations, permitting the iron liberated to be demonstrated as fully as after the employment of either sulphuric or nitric acid alcohol.

The time during which these reagents must be allowed to act on a piece of tissue varies. I prefer to give general statements on this point, because specific directions are impossible in a case where the size of the object, the quantity of the reagent, and the temperature constitute the conditions. Bunge's fluid extracts as readily as it liberates the iron in thin sections of tissue, but when the latter is in mass the reagent requires a length of time which may vary from a week to two months, all depending on the size of the object and on the temperature, which in summer may be that of the room (20°—29° C.), but in the colder seasons that of the warm oven (35° C.). Sulphuric acid alcohol acts more slowly, and consequently requires a longer time for liberating the iron in unsectioned objects, while in sections its action is complete in from one to four days, this depending also on the temperature, the most favourable being 35° C. A longer stay than is just sufficient to liberate all the organic iron results in removing from the sections some of the iron set free, the more being extracted the longer the sections lie in the reagent. When examples of the Protozoa and Protophyta were subjected to

[1] These differences in extractive capacity exhibited by weak and strong alcoholic solutions of hydrochloric acid have apparently not been noted by Petit (loc. cit.), who used the diluted reagent in a Soxhlet apparatus to remove the inorganic iron compounds from barley. As the boiling-point of hydrochloric acid is higher than that of alcohol, it is obvious that little of the former must pass from the 1 per cent. solution at the bottom of the flask as vapour to condense above and act on the substance whose iron is to be extracted, while the alcohol is readily converted into vapour; in other words, the reagent in the upper part of the apparatus must be much more dilute than that in the flask below, and consequently its extractive power must be very feeble. This method is, therefore, open to the objection that it does not ensure the removal of inorganic iron compounds.

the action of the acid alcohol the full effect was obtained at the end of twenty-four hours at the latest, when the temperature was 35° C. With nitric acid alcohol the liberation of the organic iron was rapid, sections of vegetable tissue (Erythronium and Iris) giving, after a stay of ten hours in the reagent at 35° C., an intense reaction with the acid ferrocyanide mixture. At a lower temperature the result was less marked, but the reaction was deeper than that obtained with sections treated with sulphuric acid alcohol for the same length of time and at the same temperature. The process of liberation was usually completed in about thirty-six hours. So little does nitric acid alcohol extract the iron it liberates that in sections of the ovary of Erythronium americanum kept for six weeks in it I found little diminution in that intensity of the iron reaction which sections, placed in the same fluid at the commencement with the others, gave at the end of two days. With sections of animal tissue the intensity of the reaction was less marked with the prolonged stay in the reagent, which, after four or five days' action, slightly alters the cellular structures. When nitric acid alcohol is allowed to act on a section for a longer time than is necessary to set free all its organic iron, diffusion of the iron salts thus formed is apt to occur, especially in vegetable preparations, the cytoplasm giving in such cases a reaction for iron.

That the iron demonstrated after the use of acid alcohols is derived from organic compounds I have shown by numerous experiments. I have found that when thin sections of animal or vegetable tissue are covered with a large quantity of Bunge's fluid and kept for three days at 35° C. or higher, the teased-out cells give no iron reaction when mounted with glycerine and ammonium hydrogen sulphide on the slide, even after two weeks and at 60° C. Furthermore, sections so treated with Bunge's fluid, when subsequently subjected to the action of sulphuric acid alcohol or of nitric acid alcohol, yield no iron reaction whatever. Bunge's fluid, therefore, extracts the iron which the prolonged application of ammonium hydrogen sulphide and glycerine at an elevated temperature liberates and demon-

strates, and with this removal disappears the iron demonstrable after treatment with either of the other acid alcohols. This shews that the iron in such cases cannot be derived from the reagent nor from the glass of the vessel used, and this is emphasised by the results of other experiments. I extracted with Bunge's fluid all the iron from a series of sections of an ovary of Erythronium, and then subjected these to the action of a large quantity of sulphuric acid alcohol for twenty-four hours at 35° C. These gave no iron reaction, while others did so which had not been treated with Bunge's fluid, and which were put in the acid alcohol at the same time. That the absence of an iron reaction was not due to a lack of absorptive capacity on the part of the section, brought about by Bunge's fluid, was proved when such sections were allowed to stay in sulphuric acid alcohol containing a little ferric salt in solution[1] for half an hour. The reaction obtained was marked, and almost wholly confined to the nuclei. These experiments were repeated again and again with sections of animal and vegetable tissues, and the results were always the same, proving that the iron demonstrable after acid alcohol has been used on tissues is derived from the latter, and not from the reagent or the vessel used. These experiments indicate, however, how necessary it is, in investigating the distribution of iron in tissues, that the reagents should be absolutely free from iron, and that, in sections of tissues containing iron in an inorganic or albuminate form, there is danger, when either sulphuric acid alcohol or nitric acid alcohol is used upon them, of its redistribution, and especially of its deposition in those parts of the cell which absorb various compounds readily. In order to guard against this, I found it advisable to steep the sections in a quantity of Bunge's fluid for a time which varied with the temperature at which the reagent was applied, as, for example, for one to two hours at 50°—60° C., but for eight to ten hours at 35° C.

[1] This solution was made in the following way. A quantity of sulphuric acid alcohol was allowed to act on ferric oxide in powder for about a week, when a portion passed into solution as a ferric salt. Of this solution 1 c.c. was taken and added to 10 c.c. of pure sulphuric acid alcohol.

Bunge's fluid extracts very little or no iron from sections when the temperature is below 20° C., but at the higher temperatures stated the extraction is complete at the end of the intervals mentioned, and with a longer action more or less of the "masked" iron is liberated and removed. When a tissue—as, for example, that of the spleen in some animals—contains an excess of iron in an inorganic form, the time of extraction must be prolonged, and the extracting fluid large in quantity. After the inorganic and albuminate iron has been thus removed from a section—a result which may be demonstrated by treatment of the preparation with ammonium sulphide,—it may be subjected to the action of either of the two other acid alcohols to liberate that portion of the "masked" iron as yet unaffected.

The acid alcohols do not readily attack and liberate the iron of hæmoglobin and hæmatin except at a high temperature. Of this fact I have convinced myself by numerous experiments on hæmoglobin, whether prepared from alcohol material or from that coagulated by heat. A quantity of it, in a powdered form, put into a flask and covered with a quantity of Bunge's fluid, was heated for twenty minutes, and the fluid then, after filtration through a filter free from iron, was neutralised and treated with ammonium sulphide. The mixture gave no immediate evidence of the presence of iron, but when the test-tube containing it was put aside for twenty-four hours, a dark-green sediment made its appearance, and this was shown to be sulphide of iron when it was separated on an iron-free filter and treated with a quantity of an acid ferrocyanide mixture. This iron was, in great part, derived from the hæmoglobin and hæmatin, as well as from organic combinations present in the leucocytes and plasma, and but little had its source in the inorganic and albuminate compounds of the same, a fact shown by further experiments on the powder which had once been acted upon by boiling Bunge's fluid. The extract made with a fresh quantity of the reagent gave, on neutralisation and on the addition of ammonium sulphide, the same evidence of the presence of iron that was obtained in the first experiment. A

third, fourth, and fifth extraction resulted in the same way. When, on the other hand, a quantity of crystallised hæmoglobin was acted upon by the reagent for forty-eight hours at 35° C., the filtered fluid, tested for iron in the manner described, gave a scarcely appreciable evidence of the presence of the metal. The iron, therefore, which is found in animal tissues after the use of Bunge's fluid at either 35° or 50° C. for short intervals cannot very well be supposed to be derived, in any appreciable quantity, from the hæmoglobin in them, and as ammonium hydrogen sulphide does not affect the iron of the pigment, yet reveals the iron of "masked" combinations of an apparently less firm character, it follows that weak solutions of hydrochloric acid at slightly raised temperatures must attack such combinations more readily than it affects hæmoglobin. This was most clearly shown by results of experiments on hæmoglobin and chromatin with a quantity of Bunge's fluid for twenty-four hours at 35° C. When hæmoglobin alone is thus treated, neither the powder nor the extract gives any appreciable indication of free iron, but the latter is readily demonstrable in chromatin, or in mixtures of chromatin and hæmoglobin, after similar treatment. Since the iron in hæmoglobin is not affected to any perceptible degree by treatment with the reagent for twenty-four hours at 35° C., one may postulate that it is as little affected by treatment with either of the other two acid alcohols at the same temperature, and experiments with these have given results which bear out this conclusion.

The substance chlorophyll, the relations of which to iron, though generally recognised, have not been definitely determined, is, as is well known, an abundant constituent of the cells in many vegetable forms, and, therefore, a brief discussion of the possibility that this substance is the source of the iron demonstrated in vegetable cells, is necessary.

Some of the more recent investigators of this substance have made conflicting statements on the question of the presence of iron in the molecule. Adolph Hansen[1] found it to contain

[1] 'Die Farbstoffe des Chlorophylls,' Darmstadt, 1889, p. 58.

iron, while Emich, at the request of Molisch,[1] examined a quantity of pure chlorophyll and found it free from iron. Molisch also made observations on the subject, and determined that, after every care was taken to prevent contamination with iron salts through impure extracting fluids, the ash of chlorophyll gave not the slightest reaction for the metal. Gautier[2] also claims that it does not contain iron. Schunk,[3] on the other hand, found ferric oxide in the ash of phylloxanthin, one of the decomposition products of chlorophyll, even after that compound had been treated with acids and after repeated solution of it in ether.

The material from chlorophyll-holding organisms was, in all cases, thoroughly freed from that substance before the disposition of the iron in it was examined. Chorophyll, however, has not in any of my preparations yielded any evidence that it contains iron, nor does its presence or absence at all affect the question of the occurrence of iron in other compounds in the cell. This is very distinctly shown when one compares the results, obtained from experiments on vegetable cells holding chlorophyll, with those determining the distribution of iron in Fungi and in Monotropa uniflora and Corallorhiza multiflora, which are destitute of chlorophyll. In the two latter the disposition of the assimilated iron is as it is in the chlorophyll-holding Phanerogamous plants, and consequently one may dismiss the objection that the pigment constitutes the source of the iron demonstrated by my methods in the nuclei of vegetable cells. It may be proved also from Monotropa[4]

[1] Op. cit., p. 87.

[2] 'Chemie Biologique,' Paris, 1892, p. 20.

[3] "Contributions to the Chemistry of Chlorophyll," No. 4, 'Proceedings Roy. Soc.,' vol. l, 1891, p. 302.

[4] The importance of Monotropa material for control purposes renders a short description of the methods of preparation employed upon it necessary. This plant, when hardened in alcohol, blackens more or less through the production on the part of the dying cells of a dark greenish-blue pigment, but it remains colourless when fixed in solutions of corrosive sublimate, a reagent whose use is, for reasons already mentioned, objectionable when ammonium sulphide is to be employed. To obtain material on which this reagent may be

that none of the iron found in the nuclei is derived from the cytoplasm, for there is very little and often no cytoplasm in the cells of the coats of the ovules in this plant, and yet the nuclei of these give as intense a reaction as those of the ovary of Erythronium, Iris, Hyacinthus, or any form in which the cytoplasm is abundant.

In order to get the best results with the use of the acid alcohols, I have found that the tissues must be well hardened. If the tissues are fresh or imperfectly hardened, the application of acid alcohols for a time sets free the organic iron, but the structure of the cellular elements is more or less changed in such cases by the acids—a change not at all found to occur when the tissues have been carefully hardened. Strong alcohol (90—95 per cent.) was used for this purpose, and it was found to present, over the other hardening reagents, a number of advantages. It can by redistillation be made free from iron, and when it is of absolute strength it neither extracts any of the iron compounds (hæmatins excepted) from tissues, nor allows these to diffuse. There is the important point also, that tissues fixed with it can be subjected to all the reactions for iron, without incurring the risk of complications due to the deposition of iron or other metallic salts, which occur when other hardening reagents are used. In this way one may treat pieces of a tissue with ammonium hydrogen sulphide and with the acid alcohols, and thus allow the methods to control each other. When, on the other hand, it was not necessary to use ammonium hydrogen sulphide on the tissues, other hardening reagents were employed, but only such as did not, by their presence in the tissues, interfere with or obscure the demonstration of the iron. Saturated solutions of corrosive sublimate and ½ per

used advantageously, parts of the fresh plant are thrown into boiling distilled water, and those which remain uncoloured at the end of ten minutes are further hardened for several days in absolute alcohol. I have often treated material so prepared with the warm glycerine and sulphide mixture for from four to ten days, and then with an acid ferrocyanide solution converted the ferrous sulphide demonstrated into Prussian blue. Such preparations are probably the most instructive obtainable in regard to the question of the relation of iron to the vegetable cell.

cent. solutions of osmic acid were found serviceable, the latter reagent also having been used in the combination known as Flemming's fluid.

The corrosive sublimate solution was allowed to act on the preparations of tissue for about ten minutes, after which they were washed for a few minutes in distilled water, and then in 50 per cent. alcohol. The hardening was completed with alcohol of 70 and 90 per cent. strengths in the usual way. When Flemming's fluid was used the tissue was not allowed to lie in it for more than half an hour, while for the osmic acid solution not more than ten minutes were given, and the fixation was carried on further with alcohol of 50, 70, and 95 per cent. strengths. Preparations, whether made with corrosive sublimate or with osmic acid solutions, retain, even after careful washing, traces of the metallic salt of the reagent used, and the black or dark reaction which they give with ammonium hydrogen sulphide, in consequence of the presence of such metals, interferes with the proper demonstration of the distribution of iron by that reagent. On this material the acid alcohols only were used, and the preparations were subsequently treated with the acid ferrocyanide mixture, the Prussian blue reaction obtained not having been in the least affected by the presence of minute quantities of the metallic salts of the hardening reagents. The latter were free from iron salts, a fact of which I convinced myself by qualitative analyses.

To the use of all hardening reagents other than alcohol there are objections. Those which contain an acid may assist in the diffusion of iron salts in the tissues, and cause the deposition of these in some other parts than those in which they originally were held. Further, the acids of some of the reagents (e. g. acetic acid in Flemming's fluid) may liberate the organic iron, which cannot in such a case be distinguished from the iron of inorganic or albuminate combinations. For these reasons I have used acid hardening reagents but occasionally, and then the time allowed for their action was short, in order to reduce to a minimum the risk of liberating organic iron, and of the diffusion of iron salts through the tissues. Against corrosive

sublimate as a hardening reagent, which I have frequently used, it may be urged that it possibly assists in the diffusion through the tissues of the inorganic compounds of iron, and that consequently the distribution of the latter, in preparations thus hardened, may not correspond with that obtaining in the fresh tissue. Where this is not under investigation it is a matter of no importance, for treatment of sections of the tissue with warm Bunge's fluid for a few hours removes such compounds, and the sections so treated may be subjected to the action of the various reagents described to demonstrate the organic iron; but when it is desired to study the distribution of both classes of iron compounds in a tissue, the objection urged would, if well founded, exclude corrosive sublimate as a hardening reagent for this purpose. My experiments in relation to this were made on pieces of the same organ (liver and kidney of guinea-pig and of Amblystoma) hardened with alcohol alone, and with corrosive sublimate and alcohol, and I have found, on comparing the distribution of iron in both series of preparations, that though the possibility of the diffusion of iron salts is not excluded when corrosive sublimate is used, yet no appreciable evidence of it was manifested in the preparations. I have not, however, based my observations in any one case alone upon material hardened in corrosive sublimate, but have used material hardened in alcohol in all cases to control the results obtained when that reagent was used.

When the iron was liberated by acid alcohol the whole of it appeared as a ferric salt in some tissues, while in others a very small portion of it also was set free as a ferrous compound.[1] The latter condition was illustrated in some of the Protozoa. In such preparations all the iron set free is demonstrated, after treatment with ammonium sulphide, as a ferrous salt, and the preparations may then, on being acted upon with a mixture of equal volumes of dilute solutions of hydrochloric acid and potassic ferrocyanide, reveal all their liberated iron as Prussian blue. The iron in the ferrous form is usually so very minute in quantity, if present at all, that it may be

[1] For an explanation of the preponderance of the ferric compound see p. 268.

neglected in the making of permanent preparations. In order to prevent contamination of the sections with iron compounds in the demonstration, the solutions of potassic ferrocyanide were, on all the occasions used, not more than a week old, although I found that those of longer standing, up to the end of two months or so, when filtered carefully, gave preparations which were free from any objectionable characters. The strength employed was 1·5 per cent., and a volume of this was mixed with an equal volume of hydrochloric acid of 0·5 per cent. strength, when the mixture was required.

The sections, after removal from acid alcohol, were first washed in pure alcohol, then in distilled water, after which they were placed in the acid ferrocyanide mixture for not more than five minutes. Again washed carefully in distilled water, they were either dehydrated in alcohol, cleared in oil of cedar, and mounted in benzole balsam, or, before they were put through this course, stained with either safranin or eosin. The staining reagents were of 1 per cent. strength in 30 per cent. alcohol, and the time allowed for the action of the eosin was three minutes, while that for the action of safranin was half an hour. The excess of the stain in either case was removed with alcohol. The advantages given by the use of these stains I have explained in the description of the constituents of the nucleus. Very frequently I have found that a preparation which illustrated, in a remarkable way, some point in the distribution of iron in the cell, became useless through a complete fading out of the blue. The causes of this result are two: exposure of the preparation to the light for a time, and the use of inferior oil of cedar, that is, impure through the presence of minute quantities of water and other matters. I found that when I used old oil of cedar to clear up the sections and to remove all traces of alcohol, the preparations would keep their beauty unimpaired, if placed away from the light in the slide box. In some way the preservation of the blue colour depends on leaving a trace of the oil used in clearing-up upon the section when the balsam is added, but in this quantity allowed to remain there must be no alcohol or

water. When oil of cloves or oil of lavender was used all the preparations faded, for some reason at present unexplainable. The presence of safranin or eosin in the preparation does not influence, in any way, its chances of fading, but if the excess of the stain has not been removed it is apt, while the balsam is hardening, to diffuse, and thereby obscure the finer details of the preparation. That it is not difficult to keep Prussian blue preparations of animal and vegetable tissues, if carefully made, is shown by the fact that I have had now for over two years several hundred of such which retain unimpaired the original intensity of the reaction.

I have always washed the sections with distilled water, before putting them in the acid ferrocyanide mixture, because the presence of acid alcohol, especially that containing nitric acid, causes decomposition of the ferrocyanide and a deposition of Prussian blue in parts of the preparation in which iron did not occur originally. The acid ferrocyanide mixture itself decomposes after twenty minutes with the formation of Prussian blue, but that this is not, even in an infinitesimal part, the source of the blue that obtains in a section during the first five minutes after the mixture is made, was shown by the complete absence of a blue reaction in other sections of the same tissue (*e.g.* cartilage, muscle, ovary of Erythronium) placed in the mixture at the same time without having previously been treated with an acid alcohol. The distribution of the Prussian blue due to such a decomposition is quite different from that which one finds in preparations treated with acid alcohol, but in which this decomposition was avoided, for when one leaves sections of animal or vegetable tissue in the acid ferrocyanide mixture for two hours, the blue colour is uniformly diffused through the section, not localised as it is when the reaction is due to the iron of the tissue.

In the permanent preparations made to illustrate the distribution of iron, and on which no staining reagents were employed, the parts revealed by the transparent blue are not as sharply outlined as they would be if stained with hæmatoxylin for example, owing to the cytoplasmic parts, over or under the

structures coloured blue, obscuring the latter. This may be obviated, especially with high powers, by raising the Abbé condenser to the level of the stage and removing altogether its diaphragm, when the brilliancy of the light in the field of the microscope enhances the blue due to the iron reaction, while it renders more or less obscure the other details of the preparation. It was only in this way that I was able to determine the occurrence of very minute traces of iron in the tissues and, when the sections were stained with safranin, of bodies which gave but a feeble Prussian blue reaction (figs. 45 and 46).

The sections of tissue were made, either by the free hand with a polished steel knife, or by the paraffin or celloidin methods. Care was taken that the knife should not yield a trace of iron to the sections. When the paraffin method was employed the surface of the cutting instrument was dry, but with the other methods it was covered with absolute alcohol. The transference of sections from one fluid to another was done with goose-quill points or with glass needles.

I may not leave this part of the subject without a reference to the potash method for the liberation of "masked" iron, as described by Molisch, but afterwards determined by him to be untrustworthy. I have studied the effect of concentrated solutions of potassium hydrate upon vegetable tissues hardened in alcohol, and have obtained, frequently, evidences of the presence of iron in the cell wall, cytoplasm and nucleus, but the amount thus indicated in the last was always very much less than could be demonstrated by the other methods, while the reagent so altered the nuclei that a determination of the definite relations of the iron observed to the nuclear structures was impossible. My observations have convinced me that a very large part of the iron demonstrable after the use of this reagent is derived from the latter, however pure it may apparently be, and I am, therefore, upon this point in accord with Molisch. One of the readiest ways of proving this is by extracting all the iron from sections of vegetable tissues by keeping them in a quantity of warm Bunge's fluid for several

days and then transferring them to a quantity of a concentrated solution of potassium hydrate for an hour, after which interval the sections give abundant evidence of the presence of iron. That not a trace of iron is left in the sections by Bunge's fluid may be shown by incinerating some of the sections so treated and examining micro-chemically the ash for the presence of iron. Were one able to obtain the reagent absolutely free from iron, its employment for this purpose, limited as it must be through its drastic action on cellular structures, would, however, still be open to objection on the score that it dissolves and redistributes the iron of the tissues.

III. General Observations on the Distribution of Assimilated Iron in highly specialised Animal and Vegetable Cells.

The greater part, and sometimes the whole, of the assimilated iron in the cells of the higher forms of animal life is held in the nucleus, in the chromatin of which it is chiefly found. The chromatin fibrillæ, the chromatin granules, the nodal points of the chromatin network, all exhibit, after the employment of the methods described above, the clearest evidence of the presence of iron. Though no definite comparison is possible, yet, judging by the depth of colour resulting from the Prussian blue reaction in a large number of animal nuclei, one may say that the amount of iron thus demonstrated appears to correspond in all cases with the amount of chromatin present. This is probably best seen after the use of sulphuric acid alcohol, followed by treatment with an acid ferrocyanide solution, the sections thus prepared being compared with others simply stained with a reagent like Ehrlich's hæmatoxylin so employed as to affect the chromatin only. In this case the hæmatoxylin stain in the chromatin is always found to correspond in intensity, in the object stained and in the general distribution of the stain, with the blue reaction obtained in the other sections. If, further, sections

illustrating the Prussian blue reaction, be stained also with safranin, which, when carefully employed, affects only the chromatin, it will be observed that all the elements coloured by the safranin exhibit the blue reaction also, the combination of the red and the blue giving to the chromatin a colour of a violet shade (figs. 46 and 48).

It is not, however, the chromatin alone in the animal nucleus that possesses assimilated iron, for one sees in sections exhibiting the Prussian blue reaction, but more readily in those which have been also stained with safranin, that nucleolar elements possess a light blue colour (figs. 45 and 48 *a*). Some difficulty is experienced in observing this under ordinary conditions, but this is overcome, when homogeneous immersion apochromatic objectives are employed, by withdrawing the diaphragm of the Abbé condenser, the great amount of light thus transmitted causing all the blue parts to appear with remarkable distinctness, and amongst these the nucleolar bodies coloured light blue, while all the other elements are rendered indistinct or invisible. When, however, safranin has also been employed to stain such preparations, the chromatin absorbs it but the nucleolar elements are absolutely unaffected by it, and they thus stand out in marked contrast with the other structures. Such nucleolar bodies take but a faint stain with hæmatoxylin, a fact which, considered in connection with the result of the employment of safranin, would seem to demonstrate that they are not essentially formed of what the cytologist comprehends under the term chromatin. The number of these in a nucleus varies, and the shape and size of each are not constant, while not unfrequently the central portion appears free from iron, the outer or peripheral part, coloured light blue, appearing as an envelope of greater or less thickness for the uncoloured part (fig. 46, *a* and *d*). These bodies are always attached to the chromatin network, and sometimes there appears about them a membrane derived from, and continuous with, the fibrils with which they are connected. This is very distinctly seen in the safranin preparations, the membrane in this case exhibiting a combination

of the blue and red reactions, and thus appearing in sharp contrast with the enclosed nucleolar body coloured light blue.

It is chiefly in the nuclei of the glandular cells that one finds these nucleolar bodies, and they are most distinctly seen in large nuclei, as, for example, those of hepatic and renal cells and of the intestinal epithelium of Necturus lateralis. They are very rarely seen in the nuclei of the muscle fibre and in those of the cutaneous epithelium of the same animal, while they are never present in those of leucocytes or lymph cells, or in those of the red blood-corpuscles. In the search for them in all these elements the greatest assistance is obtained from the employment of eosin, which, in sections exhibiting the Prussian blue reaction, gives these bodies an ochre-red colour, while the parts showing a dark-blue reaction are unstained by it (fig. 47). In the nucleus of the glandular cell which is passing into the mitotic phase, the nucleolar body disappears, apparently by solution into the chromatin threads, for in the nucleus of a renal cell, in which the meridional disposition of the chromatin filaments obtained preparatory to the formation of the loops, I saw, attached to one of the filaments and partly embraced by its substance, what appeared to be the remains of such a body. In later stages of mitosis not the slightest evidence of this body or of its remains can be observed.

Whether the iron which these bodies contain is that of a small quantity of chromatin dissolved in them, I am unable to say. The fact that they take sometimes a very feeble stain with hæmatoxylin, seems to indicate that they may contain a small amount of chromatin. The iron in them is held neither more nor less firmly than in the typical chromatin elements, since in hepatic nuclei containing them, prolonged treatment with ammonium hydrogen sulphide in the warm oven does not result in demonstrating any difference, except in the amount of iron in the one and in the other. The substance which holds the iron does not possess the slightest affinity for safranin, but attracts eosin as no other cellular constituent does, and in these properties, as well as in the

very small amount of iron present, there would appear to be distinctions which separate it from chromatin. My preparations were chiefly obtained from the organs of the fasting animal, and as I did not succeed in my attempts at feeding artificially some examples of Necturus that I had, it is not possible for me to say whether the constitution of the nucleolar bodies is always similar to, or ever different from that described; but in preparations of the liver and other organs of specimens of Amblystoma punctatum killed soon after their capture, or after artificial feeding, the nucleolar bodies appeared to present the characters noted in the cells of the fasting animal, the smaller size of the elements in this case, however, not allowing as clear a view of them as was desired.

In the nuclei of the liver-cells of Necturus, as illustrated in preparations made after the manner described, I frequently found a third element, whose significance is unknown to me. It manifested itself by the red stain which eosin gave it, the nucleolar bodies taking, in contrast, an ochre-red stain. It had no constant shape or form, in some cases being of a filamentous character, in others resembling a localised granular deposit (fig. 47); and when the structures were filamentous, several usually appeared in the same nucleus. The substance forming them did not contain the slightest trace of iron, and therefore appeared to have no relation to the nucleolar bodies or to the chromatin. I have not in any other organ observed similar structures.

The disposition of the iron-holding compound in the nuclei of Amphibian ova deserves special mention. In the ovarian ova, whose nuclei contain no peripheral nucleoli, the iron is distributed as represented in fig. 36, the chromatin in this case forming a fine reticulum, in the trabeculæ of which large granules are found with lateral prolongations. The iron demonstrated in this preparation was set free by sulphuric acid alcohol, but a disposition of iron in the main like this may be found in similar nuclei when the latter are, on removal from the ova, broken into small pieces on the slide in the glycerine and sulphide mixture, and, thus prepared and provided

with a cover-glass, kept for days in a warm oven. This method must be resorted to in order to get the iron reaction, since otherwise the large nuclei may be kept for a month in contact with the reagent in the warm oven without resulting in demonstrating, in the slightest, any iron reaction. In the peripheral nucleoli, when they are present, the amount of the iron, as indicated by the depth of the reaction, is great, but in the remaining elements of such nuclei it is small. When such preparations are examined with a strongly magnifying objective, the chromatin network, as revealed by the iron reaction given, is found to be less distinct, and instead of granules of iron-holding substance arranged at definite positions along the course of the fibrillæ, as in ova much less developed, the iron is now seen to be chiefly confined to beadlets, few in number, sometimes regularly, sometimes irregularly, disposed on the fibrillæ, which, in ammonium hydrogen sulphide preparations, manifest but a feeble greenish tint. There is an inverse relation between the size of the nucleoli and the amount of the chromatin in the network, and an examination of some nuclei in which the formation of the peripheral nucleoli has commenced, and of those in which the development of these bodies is much more advanced, irresistibly suggests that the latter are derived from the chromatin of the network. I have elsewhere[1] pointed out that the solution of the substance of which these nucleoli are composed and its diffusion from the nucleus into the cytoplasm of the ovum are connected with the formation of the yolk-spherules in Amphibia. That a solution of the peripheral nucleoli takes place has been noted by O. Schultze[2] and Born.[3] Schultze found that with the solution of the nucleoli (keimkörperchen) the contents of the nucleus and the substance surrounding the latter were affected in the same way by reagents and staining fluids, and he believed that the dissolved sub-

[1] 'Transactions of the Canadian Institute,' Toronto, vol. i, part 2.

[2] "Untersuchungen über die Reifung und Befruchtung des Amphibieneies," 'Zeit. für wiss. Zool.,' vol xlv, p. 177.

[3] "Die Struktur des Keimbläschens im Ovarialei von Triton taeniatus," 'Arch. für Mikr. Anat.,' vol. xliii, p. 1.

stance diffused from the nucleus of the ovum into the cell-body. Born observed that the nucleoli are always placed as closely as possible to the cell protoplasm, while the chromatin in the development of the nucleus and ovum becomes so finely divided in the karyoplasm that it is stainable with great difficulty, and it is as difficult to demonstrate optically, a condition which continues till the formation or deposition of the yolk-grains (Dotterkörner) commences. In later stages the persisting peripheral nucleoli lose their capacity for absorbing colouring matters.

In support of these observations of Schultze and Born, I may but add that the iron in the cytoplasm of the ovum makes its appearance only after the solution of the peripheral nucleoli commences. The substance forming the peripheral nucleoli does not react with staining reagents as does the chromatin of the nuclear network, and especially with the indigo-carmine staining fluid of Shakespeare and Norris the resulting stains of each are different, the chromatin of the network being coloured red while the nucleoli are stained blue or green, the latter colour obtaining also in the yolk-spherules of such preparations. A further difference is noticeable in the effect that ammonium hydrogen sulphide exercises when applied to these structures for some time at an elevated temperature. In this case the iron of the peripheral nucleoli reacts more readily than that of the chromatin of the network, but less readily than that of the yolk-spherules, which in the ova of Necturus and Amblystoma give a green reaction in a few minutes with the reagent. It would appear as if the iron compound undergoes a change in its transference from the nucleus to the cytoplasm.

The peripheral nucleoli appear to be formed at the nodal points of the chromatin network, if one may judge from preparations of which fig. 34 is an illustration; but there is a possibility that these represent a pathological condition, since they are not common in the ovary when, if they were normal, they should be present in larger numbers. I have, moreover, found that they were accompanied by examples of

another condition which I regard as pathological. In the latter the nuclei were indistinct or disintegrated, their chromatin had disappeared, and the surrounding connective tissue, with its blood-vessels and their red corpuscles even, gave in a few minutes, with warm ammonium hydrogen sulphide, an iron reaction, frequently so deep as to obscure largely the details, while the tissues, a little further away from such examples, and other ova under exactly the same conditions of treatment with the reagent, gave no such reaction. It may possibly be that the chromatin of such disintegrated ova furnished the iron observed thus diffused in the connective tissue and blood-vessels.

In the nuclei of all the higher vegetable organisms the assimilated iron compounds are, on the whole, distributed as they are in the nuclei of the more highly developed animal forms, a fact which may be demonstrated in any Phanerogamous plant, especially readily if its nuclei are large, as is the case in Erythronium americanum. In many of the preparations of the latter form the chromatin filaments were, in the process of teasing-out, partially or almost wholly set free from the nuclei containing them, and to the parts thus set free, as well as to the remainder, the glycerine and sulphide mixture always gave a distinct reaction for iron in a few days (fig. 17). Mitotic figures in such preparations appeared very sharply defined through the iron thus revealed in the chromatin elements. In successful preparations made by this method the reaction for iron is very marked, as much so as in those made with sulphuric acid alcohol; and in this respect there is a contrast between animal and vegetable nuclei, for in the former the glycerine and sulphide mixture brings out, after a longer application and less frequently, a reaction as intense as that which may be obtained after treatment of the nuclei with acid alcohol.

Of nucleoli and nucleolar bodies there are at least three kinds. The reaction for iron given in one variety by the glycerine and sulphide mixture was weak, and it was obtained at the same time that it appeared in the chromatin network or filaments. These are smaller, apparently, in the hardened

than they are in the living cell, for, as a rule, they only partially occupy the cavity in which they lie (figs. 17 and 19). I have in some cases isolated them from their nuclei in the glycerine and sulphide mixture, and the greenish reaction which they gave could, therefore, not have been due to the iron of a compound which diffused from the chromatin elements into them. When the sections were treated with sulphuric acid alcohol and subsequently with the acid ferrocyanide mixture and the eosin solution, the result was usually that of which fig. 42 is a representation. These nucleoli stain intensely with eosin, which also colours very slightly the chromatin network, the blue of the latter thereby becoming violet, but after being thoroughly washed in alcohol the bright blue colour returns; while this treatment makes no difference in the intensity of the stain in the nucleolar bodies. These effects are most distinctly observed when the diaphragm of the Abbé condenser is removed from the field, in which case it is possible to see the most minute of the nucleolar elements, a device that is necessary when the nuclei of ordinary parenchyma cells are under examination.

In the second class are those nucleolar elements which may be found in the cells of the nucellus, and which are composed of chromatin, since they give a deep iron reaction after the employment of any of the methods of treatment for liberating the element, and since, also, they stain in every respect like the chromatin threads. They usually occupy cavities in the nuclei like those which contain the eosinophilous nuclei last described. I regard these as reserve masses of chromatin deposited in the nuclei engaged in the formation of chromatin, which eventually is transferred to the cells of the endosperm. To this subject I propose to refer again.

Nucleoli of the third class are to be found in the nuclei of the embryo-sac (fig. 44, *a* and *b*). They are not present in the mitotic nucleus, but in the retrogressive stage they appear on the course of the filaments as spherical elements enclosing one or more refracting corpuscles and containing but a small amount of iron, which, however, in later stages, when the fila-

ments became thinner and less rich in chromatin, is more abundant. These nucleoli are eventually formed chiefly of chromatin, and in stained preparations appear to contain nearly all the chromatin of the nucleus. When mitosis again commences the filament forms at their expense, the increase in size of the filament keeping pace, apparently, with the decrease in the quantity of chromatin which the nucleoli contain. Finally, before their disappearance, when they contain but a minimal quantity of iron, they take the eosin stain deeply.

All these forms of nucleoli take up safranin from solutions as readily as do the chromatin elements in the same nuclei, and they hold the stain as tenaciously when they are washed with alcohol. They are in this respect different from the eosinophilous nucleoli in the animal cell, which appear to be unrepresented in the vegetable cell.

Of an exceptional character are the nucleoli in Corallorhiza multiflora and in Spirogyra. In these the greater portion of the chromatin in each nucleus forms a single large spherical element unconnected with the chromatin network, which after prolonged treatment with the glycerine and sulphide mixture, gives a pronounced reaction for iron.

I have, on a few occasions only, in preparations illustrating the iron reaction, seen the chromatin localised at points along the course of the filament, and concluded that this was not due to faulty methods of manipulation, for hæmatoxylin and other dyes just as infrequently render such a distribution visible. It was also, with the aid of the acid alcohols, found that in the loops of the mitotic nucleus of the embryo-sac the chromatin is disposed under the membrane enclosing the filament, in such a way as to make the latter appear as a tube of chromatin.

In some of the elongated oval nuclei of the nucellus and of the fibro-vascular bundle of the ovule, Mr. Bensley has observed a point of some interest. This consists in the occurrence in the karyoplasm, amongst the trabeculæ of the chromatin network in one end of such a nucleus, of an iron-holding compound with all the characters of chromatin, and,

in some cases, in such abundance as to obscure the outlines of the trabeculæ. He has found that in the fibro-vascular bundle this end of the nucleus is directed toward the base of the ovule, and is of the opinion, as a result of some investigation of this subject, that the phenomenon in question is connected with the processes of the formation of chromatin, which he regards as taking place here.

The presence of assimilated iron, apart from its occurrence in hæmoglobin and hæmatin, is an exceptional feature in the cytoplasm of the cells of the higher forms of animal life, but the exceptional instances are themselves of a constant character, and comprise, in addition to yolk-holding ova, the cells of yolk-holding embryos, the hæmatoblasts of Vertebrates, and the ferment-forming gland-cells of all descriptions.

The iron in the yolk of Amphibian ova is held in the yolk-spherules, which manifest a strong affinity for dyes, and are usually homogeneous in composition. These give with ammonium hydrogen sulphide a dark green reaction, which makes its appearance sometimes in a few minutes, but at the latest in a few hours, when the preparation is kept warm. The reaction is uniform throughout each spherule. The enclosing cytoplasm does not, before development of the ovum begins, contain any assimilated iron; but in the developing embryo, with the multiplication of the cells and the partition of the yolk, the spherules gradually undergo solution, for they become smaller in size, and then one obtains an iron reaction in the cytoplasm of each cell. The solution of the yolk-spherules may be studied also in preparations made with the carmine-indigo-carmine fluid, the reagent giving, in the earliest stages of the embryo, a green colour to the yolk-spherules, and a red stain to the cytoplasm and nuclei; but in later stages the red colour is rarely obtainable, and both cell and nucleus, the latter especially, are coloured blue-green or dark green. This result is brought about by the solution of the yolk-chromatin in each spherule and the diffusion of the dissolved substance through the cytoplasm and nucleus of each spherule-holding cell, for in those examples of larval Amblystomata which yield pre-

parations giving a dark green or blue-green colour in cell and nucleus after treatment with the reagent mentioned, the cells are found, after the prolonged application of the glycerine and sulphide mixture, to exhibit an iron reaction in the cytoplasm apart from the spherules, and a similar reaction diffused in the karyoplasm independent of that manifested by the chromatin network, the intensity of the reaction corresponding in each case to the depth of the green stain given these elements by indigo-carmine reagent. When in the advanced development of the larval Amblystomata the yolk-spherules disappear from the cytoplasm of the cells, the nuclei and all cells, except those undergoing transformation into striated muscle, lose their capacity for absorbing and retaining the indigo of the fluid of Shakespeare and Norris. This indicates that the yolk chromatin is changed into some other compound, and the prolonged application of the glycerine and sulphide mixture confirms this, for the cytoplasm, except in secreting cells, the striated muscle-fibre, and in the hæmatoblasts and red corpuscles, is destitute of iron compounds, while the nuclei give, much more slowly, and apparently with greater difficulty, a reaction for iron which is, in contrast with what is observed in the earlier stages, confined to the nuclear network and nucleoli. The iron-containing substance is transferred to the nuclei, and with this transference the iron becomes more firmly combined—a process the very reverse apparently of that which is illustrated in the formation of the yolk-spherules, for the iron compound of the latter, though derived from the nucleus of the ovum, is less firmly combined than that of nuclear chromatin giving origin to it.

The yolk-spherules of the hen's egg, as is well known, have characters differing from those of Amphibian ova, but the most marked difference consists in the distribution of the iron-containing compound. The yolk-spherule in the ova of Amblystoma and Necturus is homogeneous, and the iron compound is uniformly distributed through it; but in the hen's egg elements of this character are to be found only in the constituents of the "white" yolk and in some of the "yellow" spherules in

the most peripheral layers of the yolk, while in all the other spherules the distinctive feature is the disposition of the iron compound in a finely granular form. This cannot be determined with fresh yolk, for when treated with ammonium hydrogen sulphide the greater part of it dissolves, and the solution becomes dark green owing to the formation of sulphide of iron. Under the microscope no formed elements can be observed in such a preparation, except those derived chiefly from the "white" region, and it is not possible to ascertain, under these conditions, the relations of the iron-holding nuclein in other parts of the yolk. Another difficulty experienced in dealing with fresh uncoagulated yolk is that, when removed from the egg the spherules disintegrate, the granular contents escaping and obscuring more or less the characters of the other elements. To avoid this the substance of the spherules must be coagulated, and to accomplish this satisfactorily I placed the eggs in boiling water for ten minutes. The spherules were thus fixed in polyhedral form, and, after these had lain in strong alcohol for several days, it was an easy matter to determine the distribution of the iron in them.

The results obtained were according to the variety of spherules examined. In those known as "white" the reaction for iron was very distinctly obtained, but it was wholly confined to their homogeneous spherical bodies. The reaction is, immediately after the application of the glycerine and sulphide mixture, light green, but this becomes deeper after a few days, when the preparation is kept at a temperature of 60° C. The homogeneous elements undoubtedly contain a quantity of nuclein, for they resist the action of artificial gastric juice and dissolve in weak alkalies, while they constitute the only part of the "white" spherules that possesses, like chromatin, the property of absorbing and retaining colouring matters. This was found to be the case specially when the spherules, coagulated by heat, were further treated with Flemming's chrom-osmio-acetic mixture for twenty-four hours, then with alcohol, and finally with a solution of safranin. When the excess of the stain was extracted with alcohol and the spherules mounted in

balsam, it was always found that the spherical elements exhibited an intense stain, while the remaining parts of the spherules were absolutely uncoloured. I found it possible to demonstrate this and the reaction for iron in the same preparation. When the "white" spherules, fixed with heat, were kept in slightly warm sulphuric acid alcohol for twenty-four hours, their spherical elements gave, on treatment with an acid ferrocyanide solution, a Prussian blue reaction, and, when subsequently stained with a safranin solution, became violet. These results show how close is the relationship between the substance composing the spherical elements and chromatin.

A few of the spherical elements in the "white" spherules are not of the character described, for in preparations made with Flemming's fluid one finds, now and then, a spherule in which one or more large droplets of fat are demonstrated by the intensely black reaction of the osmic acid. Apart from the occurrence of these, there is comparatively little fat in the "white" spherules, a fact strikingly shown when a thin section of the hard-boiled yolk, embracing portions of the "white" and "yellow" zones, is submitted to the action of the reagent for twenty-four hours, the "white" then exhibiting a greyish appearance, while the "yellow" area is almost black.

The "yellow" spherules are also richly supplied with the iron-containing compound, but this is quite differently distributed from what it is in the "white" zone. The appearances of these are subject to a great deal of variation. Some contain only large round granules, in others the granules have a punctiform character, while in others again both kinds of elements may be mingled with minute fat droplets. Owing to differences in the specific gravity of the constituents apparently, the granules may be found, in some cases, to be gathered in one portion of the spherule, the remainder of the contents being occupied by a clear, non-granular substance of a firm consistence, a character resulting from heat coagulation. It is in such spherules as these that one determines distinctly how the iron compound is disposed, for, in those in which the granules are uniformly distributed, it is sometimes exceedingly difficult

to decide whether the iron is contained in the granules or in the extra-granular substance, so intimately are these usually intermingled. The granules, whether of the large or of the punctiform variety, always contain an iron compound, while the substance in which they are shown is destitute of this element. In demonstrating this fact the acid alcohols are of the greatest service, the glycerine and sulphide mixture, owing to the large size of the vast majority of the spherules, not being as effective in liberating and demonstrating their iron, but in the smallest spherules the complete reaction may be obtained with the mixture in four or five days. In those spherules which contain, as described, granular and non-granular portions, the granules, closely aggregated as they usually are, appear very prominent by reason of the reaction for iron which they give with both methods of demonstration.

In some of the "yellow" spherules also, after treatment with sulphuric acid alcohol, vesicles of different sizes were observed, each of which appeared to be enveloped by an iron-containing membrane-like structure. Their position near the centre of the spherule often rendered the occurrence of iron in the envelope obscure, owing to the light passing through so many iron-holding granules above and below these vesicles. What the latter contained it is not possible to say, for although fat globules of a similar size can be demonstrated in some spherules when these are subjected to the action of the chrom-osmio-acetic mixture for twenty-four hours, it cannot be demonstrated that the two classes of structures are connected in any way.[1] The difficulty lies in the fact that in order to show the occurrence of iron in the envelope, alcohol in some form must be used, and by this the fat is largely, if not wholly, removed; while in those spherules treated only with osmic acid solutions the black reaction of the globules prevents a demonstration by the Prussian blue reaction of any iron present.

[1] In another paper ("On the Absorption of Iron in the Animal Body," 'Journ. of Physiol.,' xvi, 1894, p. 268) I expressed the view that these vesicles contain fat. After a more extended study of these elements than I was able to make before that paper was published, I am doubtful of this interpretation of their structure.

Apart from the question of the occurrence of fat in such elements, there may be no doubt about the intimate association of the iron-containing substance and the fat in the spherules. Owing, however, to the size of the latter, as well as to the density of the coagulated material in them, the osmic acid used to demonstrate the fat penetrates but slowly, and when, as usually happens, fat droplets stud the periphery of the spherule, little or none of the reagent reaches its interior, which then has only a straw-yellow colour. If, however, a few spherules, coagulated with heat, are kept in a quantity of Flemming's fluid for twenty-four hours, the osmic acid penetrates the spherules in some cases and causes their granules to become brownish-black, a fact which can be most distinctly observed when the cover-glass is pressed down sufficiently to disintegrate the spherules and set the granules free. If the granules are large, the occurrence of fat in them is much less readily demonstrated, possibly because the density of such elements prevents penetration on the part of the osmic acid.

These granules are undoubtedly the source of the greater part of the iron-holding nuclein isolated by Bunge from the yolk,[1] since the "white" yolk is comparatively small in amount. Miescher[2] regarded the nuclein, which he separated from the yolk, as only in part localised in the homogeneous spherical elements in the "white" portion, and he believed that the greater part of it was derived from the granules in the "yellow" spherules, and that none of it exists in a dissolved form, a conclusion fully supported by the facts concerning the localisation of the iron.

In describing the transference of the chromatin of the spherules from the cytoplasm to the nucleus of each cell of the larval Amblystoma, reference was made to an exception in the case of developing muscle-fibre. In the cells undergoing transformation into striated fibres, some of the chromatin dissolved in the cytoplasm finds its way into the nuclei as in other

[1] "Ueber die Assimilation des Eisens," 'Zeit. für Physiol. Chemie,' vol. ix, p. 49, 1885.

[2] "Die Kerngebilde im Dotter des Hühnereis," 'Hoppe-Seyler's Med.-Chem. Untersuchungen,' 1871, p. 502.

cells generally, but the greater part appears to remain in the cytoplasm of the developing fibre, and undergoes a transformation which is one of great interest in connection with the origin of hæmoglobin. In the cytoplasm of the muscle-cells there is an abundance of yolk-spherules which, as in other cells, gradually undergo solution, the dissolved substance diffusing through the cytoplasm. When the striation makes its appearance at one side of the now elongated cell, the dissolved substance passes into the striated area, for ammonium hydrogen sulphide brings out an iron reaction in this part as readily as in the undifferentiated cytoplasm and in the spherules, but confined to the dim bands, the light bands giving no evidence of the presence of the compound. In the fibre from which the spherules have all but disappeared, and in which the striated area embraces nearly the whole of its width, the reaction with ammonium hydrogen sulphide is as distinct and as marked as in the earlier stage, and this is true also of the fibre in its final form. In this stage the iron is quickly liberated by acid alcohols, as well as by ammonium hydrogen sulphide, and its presence may be readily demonstrated by means of these reagents up to the period when all traces of yolk disappear from the cells of the larvæ. After this date the iron compound becomes firmer, or, to speak more accurately, is less readily attacked by acid alcohols or the sulphide reagent, and in the muscle-fibre finally its presence may not be shown by these methods. It is not that the iron is removed from the fibre, but that the compound containing it is transformed, in red muscles, into what is called myo-hæmatin by MacMunn, or hæmoglobin by Hoppe-Seyler and others. The latter compound can, by means of the staining [illegible] of Shakespeare and Norris, be clearly shown to be stri[illegible] confined to the dim bands, which are given a grass-green colour distinctive of hæmoglobin, while the light bands and nuclei are coloured red.[1]

[1] I have pointed out the value of the reagent in this respect in my paper entitled "Studies on the Blood of Amphibia," 'Transactions of the Canadian Institute,' vol. ii, 1893.

A similar conversion of a compound in which the iron is easily attacked by ammonium hydrogen sulphide and by acid alcohols into one from which the liberation of the iron is more difficult, obtains in the dim bands of muscle-fibre in Invertebrates (Oniscus, Chironomus, Musca), but in this case the transformation does not proceed as far as the production of hæmoglobin or myo-hæmatin, if one may judge from the absence of pigment and from the fact that the liberation of the iron, though difficult, is possible, while in the case of hæmoglobin the use of acid alcohols and of ammonium hydrogen sulphide is ineffective for that purpose.[1]

In the development of the blood-corpuscles in the larvæ of Amblystoma there is, as I have pointed out,[2] a conversion of the chromatin of the hæmatoblast into hæmoglobin, a change that is analogous to that described above as occurring in muscle-fibre. In hæmatoblasts, however, the chromatin so transformed is not directly derived from that of the yolk-spherules, as is the case in muscle-fibre, but from that compound after it is transformed into nuclear chromatin. This is very distinctly seen in sections through the aortic arches of the larvæ, which have been treated with acid alcohol to liberate the iron. In the concave side of the arches are seen hæmatoblasts in all stages of division, and in these one may, by the iron reaction, differentiate between hæmatoblasts in which there is no cytoplasmic chromatin, and those in which the cytoplasm between the chromatin loops of the mitotic figure contains dissolved chromatin to an extent varying with the example of hæmatoblast noted. This cytoplasmic chromatin does not act in the same way as ordinary nuclear chromatin does towards staining reagents, as, for example, hæmatoxylin, eosin, and safranin,

[1] The fact that ammonium hydrogen sulphide will liberate the iron from hæmatin in solution, while it does not attack the iron in the compound called myo-hæmatin by MacMunn, indicates that the latter cannot belong to the hæmatin class. Its property in this respect shows that it is related to hæmoglobin. The name given to it by MacMunn certainly appears to be a misleading one.

[2] Loc. cit.

although it has an affinity for them, and it persists with this character for a long time after the stage of the hæmatoblast is passed. I have found that in a large number of the fully-formed red cells in the spleen of the larva of 35 mm. length the disc contains a quantity of the modified chromatin, and from this the iron is readily liberated, but in later stages both the number of such corpuscles and the amount of iron in the disc which may be liberated by acid alcohols gradually diminish and disappear, the hæmoglobin of the disc not yielding its iron on the employment of such methods. The nuclear chromatin, however, of all stages of the corpuscle, readily gives up its iron, even when none can be set free in the disc.

It thus appears that the hæmoglobin of the red corpuscles and the analogous compound in muscle-fibre are formed in the same way, the only difference obtaining between them existing in the fact that the pigment of muscle-fibre does not, in its evolution in the developing ovum, comprehend a stage of nuclear chromatin. The process by which they are formed is a gradual one, and the position of the iron in the molecule is apparently changed. The latter result may be partly accounted for if we consider the composition of chromatin and of hæmoglobin. Chromatin is an iron-holding nucleo-albumin in which the iron is attached to the nuclein, while in hæmoglobin the iron is held in the hæmatin molecule, and in the transformations which result in the formation of hæmatin out of nuclein, it is but natural to expect that the relations of the iron to the molecule should change also.

In secreting cells, as, for example, those of the parotid, Lieberkünian and pancreatic glands, a certain portion of the cytoplasm gives evidence of the possession of "masked" iron. When the cells of the pancreas of an adult Amblystoma are, after hardening in alcohol, subjected to the action of the glycerine and sulphide mixture for six or seven days at a temperature of 60° C., in addition to the reaction for iron obtained in the nucleus, one is found in the cytoplasm of the so-called "outer zone," in some cases almost as marked as in the nuclear chromatin. The extent of the cytoplasm involved in the reac-

tion in all the specimens which I examined varied considerably, whether according to the stage of secretory activity could not be determined after the use of ammonium hydrogen sulphide, for this reagent, in a day or two at an elevated temperature, causes the zymogen granules to disappear; but in sections of the pancreas from the same animal, after these had been acted on by sulphuric acid alcohol, then with the acid ferrocyanide solution and eosin, the iron holding area in each cell was demonstrated by the resulting Prussian blue, while the zymogen granules were given an intense red stain, and in this case it was found that, apart from the granular zone, the cytoplasm was uniformly blue. In other conditions of activity the iron-holding area was increased or decreased in correspondence with the decrease or increase in the extent of the granular zone. In the exhausted condition of the gland-cell, that is, in which there were but few granules, arranged in the "border" fashion near the lumen of the tubule, the whole of the cytoplasm exhibited the blue reaction, but the latter was less marked than when it was confined to a narrow zone in the neighbourhood of the nucleus. The relations of the extent of the iron-holding area to the stage of secretory activity were less easy to determine in the Lieberkühnian and parotid glands, for it is not possible to demonstrate the mucigen in the former or the zymogen in the latter as prominently as the zymogen granules may be in the pancreas, but in these the iron-holding area appeared in all cases to correspond, in the main, with the "protoplasmic" or "outer" zone. In the "mucous" cells of the submaxillary gland of the cat and dog only a narrow zone of cytoplasm about the shrunken nucleus contains iron, but in the large crescents of Gianuzzi in the cat the whole of the cytoplasm is iron-holding. In the peptic tubules of Amblystoma the cytoplasm in the outer half of each cell contains iron, and this is also true of the chief cells in the cardiac portion of the stomach in the dog and cat. In the parietal cells in these animals the cytoplasm is absolutely free from iron. The iron-holding zone in each chief cell appears to vary in extent with the stage of secretion, but I am unable to speak

as definitely upon this as upon the relations, in this respect, observed in the pancreatic cells of Amblystoma, for I have not been successful in my efforts to obtain, from examples of the latter animal, preparations of the gastric glands illustrating marked variations in the stages of secretory activity, and have had to rely upon those made from the cat and dog, in which the chief cells are comparatively small and less favorable for observation on this point.

It is only in the mucous glands of the skin of Amphibia, and in the renal tubules of Vertebrates generally, that I find exceptions to the rule that glandular secretion is associated with the presence of an iron-holding cytoplasm. I have not found any exceptions in Invertebrates to this generalisation, but my observations have not been comprehensive enough on this point, and I must speak with some reserve in regard to it. In the Protozoa, as I will show further on, the presence of assimilated iron in the cytoplasm seems to be a constant feature, the iron not being confined to any part of the cell, but uniformly distributed through it, and there is a probability that this cytoplasmic iron-holding compound is also associated with the secretion of ferments functioning in the digestion of the ingested food. In the glands named above, which are mentioned as exceptional instances, the absence of assimilated iron from the cytoplasm may be explained on the ground that the secretory process of a renal cell is widely different from that of a pancreatic cell, the cytoplasm in the latter, but not in the former, elaborating a portion of its own constituents to furnish the secretion, whereas in the renal cell the process is largely one of transference only. If the explanation should hold in all possible cases of exception, then it would follow that the iron-holding compound is an important element in the elaboration of the zymogens. I have elsewhere[1] pointed out the relations that obtain between the chromatin of the nucleus and of the cytoplasm of the pancreatic cell, on the one hand, and the formative process resulting in the production of zymogen on

[1] "Contributions to the Morphology and Physiology of the Cell," 'Trans. Canadian Institute,' vol. i, part 2, p. 247, 1891.

the other; and so intimate did these relations appear that I was led to apply the term prozymogen to the chromatin. I have found, as a result of experiments on the active pancreas of Amblystoma, that the zymogen granules under certain conditions give an iron reaction. When the organ, hardened in alcohol, is put in a quantity of Bunge's fluid, and the preparation kept at the temperature of the room (20° C.) for a week, or when it is kept for two days in a quantity of sulphuric acid alcohol, teased-out portions, after the removal of the acid and on the addition of ammonium hydrogen sulphide, give preparations of which that represented in fig. 38 is an illustration. The zymogen granules give a greenish reaction, the colour making them more prominent than the other elements in the cells. The cytoplasm of the "outer zone" gives but a feeble iron reaction, and this appears only to a minor extent in the nuclear elements, both results being caused by the lessened action and feeble extractive capacity of the acid alcohols when used on the tissue in mass. When the reagents are used for longer periods than those specified the iron disappears from the zymogen granules, while it becomes more strongly marked in the nuclear elements and in the cytoplasm of the "outer zone." Owing to the effect that ammonium hydrogen sulphide exercises on the granules, causing them to dissolve or disintegrate, an effect already referred to above, it is not possible to control the results obtained with the acid alcohols by experiments with this reagent, and one may, therefore, not regard the presence of iron in the zymogen granules as conclusively demonstrated, since it may be urged that the iron reaction which they gave was due to the iron which diffused into them from that liberated in the other cellular elements. When one remembers, however, the fact that the zymogen is elaborated in a cytoplasm which is iron-holding and at its expense, the occurrence of a faint reaction for iron in the granules after the use of acid alcohols is best explained by the view that the zymogen of the pancreas contains iron, and that its antecedent, the prozymogen, is the iron-holding constituent in the cytoplasm of the "outer zone."

In the rods and cones of the retina in Amblystoma and Necturus an iron reaction was frequently obtained like that represented in fig. 37. It was always feeble and confined to the trabeculæ, which stretched across the long axis of the rod, or which formed the network in the cones. In some cases (as in fig. 37, *a*) pigment-granules were observed attached to the rods, probably derived from the cells of the tapetum nigrum, and as the pigment probably contains iron, it is uncertain whether the iron demonstrated in the rods and cones was not derived by diffusion of some iron-holding substance from this source.

The eleidin granules in the stratum granulosum in the human skin give, after treatment of sections of the epidermis with sulphuric acid alcohol, a dark green reaction with ammonium sulphide. I have not succeeded in obtaining a reaction for iron in them when the containing cells, hardened in alcohol, were simply subjected to the prolonged application of the glycerine and sulphide mixture in the warm oven. Since the chromatin of the nuclei in the underlying stratum mucosum is, as elsewhere, iron-holding, while the nuclei in the stratum granulosum are poor in chromatin, it is not improbable that the iron, at least of that part of the latter which disappears from the nuclei, is the source of the iron shown in the eleidin granules. The homogeneous substance constituting the stratum lucidum also gives a reaction for iron, which is diffuse and less marked than in the granules of the underlying layer.

In my observations on preparations of the human thyroid and of that of the dog, although it was easy to demonstrate the presence of iron in the nuclear chromatin, and to a certain extent in the cytoplasm of the cells lining the alveoli, I did not succeed in finding any of it in the "colloid" matter. Under certain conditions this substance absorbs staining matters, and it also gives[1] the molybdate-pyrogallol reaction of Lilienfeld and Monti.[2] These facts suggest that the colloid

[1] F. Gourlay, "The Proteids of the Thyroid and the Spleen," 'Journal of Physiology,' vol. xvi, p. 23, 1894.

[2] "Die mikro-chemische Lokalization des Phosphors in den Geweben," 'Zeit. für Physiol. Chemie,' vol. xvii, p. 410, 1893.

substance is allied to nuclein, and, according to Gourlay, the nucleo-albumin which he isolated from the thyroid was derived in large measure from the colloid matter which he, relying on the reaction of Lilienfeld and Monti, found to contain phosphorus. If colloid matter is therefore a nucleo-albumin, its freedom from iron renders it, in contrast with the chromatins, a subject of special interest.

Assimilated iron is rarely found in the cytoplasm of the cells of the higher vegetable organisms, and amongst the examples illustrating its presence may be mentioned the cells of the nucellus in the ovules of *Erythronium americanum*, and those of the gluten layer in the wheat-grain. The cytoplasm of the cells of the nucellus, when fertilisation has taken place, and even before this occurs, gives, after treatment with sulphuric acid alcohol, a distinct reaction for iron, which, however, in respect to intensity, is not to be compared with that manifested in the nuclei of the same cells. The iron in the cytoplasm in this case is not due to diffusion from the nuclei during the course of treatment with the liberating reagent, for it is also demonstrated in this situation in the glycerine and sulphide preparations. As the nuclei of the nucellus are much richer in assimilated iron than those of other parts of the ovule, except the embryo sac, it is possible that the cytoplasmic iron compound is *intra vitam* diffused from the nuclei, and, further, as the cytoplasm of the embryo-sac of this stage sometimes gives a diffuse reaction for iron after it has been treated with acid alcohols, its presence here may be due to a similar diffusion from the cells of the nucellus. I have observed in certain preparations in which the nuclei of the embryo-sac were in the stage of division, a large number of iron-containing granules interspersed amongst the fibrils of the achromatic spindles, and as in other preparations similar granules were stained with hæmatoxylin, like the chromatin loops, it would appear as if the granules were formed of chromatin. The cytoplasm holding these granules gave no reaction for iron.

The cytoplasm of the cells of the gluten, or so-called aleu-

rone layer (Kleberschicht) in the wheat-grain is richly supplied with a "masked" compound of iron. In some cells it is chiefly found in the large granules strewn through the cytoplasm; in others, again, apparently it is wholly contained in the latter; while in certain instances, further, it was demonstrated only in the extreme peripheral portions of the large granules. This is most clearly shown in sections of the grain after they have been treated with sulphuric acid alcohol for twenty-four hours at a slightly raised temperature. When the individual cells of other sections are treated with the glycerine and sulphide mixture for several days the reaction for iron is readily obtained in their cytoplasm, but its localisation, as observed after the use of the other method, is thus less readily determined. The "masked" compound apparently belongs to the class of chromatins, for when sections are treated with the ordinary staining reagents the cytoplasm stains deeply, especially with safranin and hæmatoxylin, and the parts which are specially affected are those which correspond with the iron-holding structures in preparations treated with acid alcohols.

Haberlandt[1] has made experiments upon the question of the site of origin of the diastase in the germinating rye-grain, and these appear to show that the ferment is elaborated in the cells of the gluten layer only. It is possible that the iron-containing compound in the cytoplasm of this layer is the zymogen or prozymogen of the ferment.

IV.—On the Occurrence of Assimilated Iron Compounds in Special Forms.

What I have said in the foregoing pages with regard to the presence of iron in the chromatin of higher forms of animal and vegetable life is true also in regard to the types of lower organisation in both kingdoms. In the investigation of the less highly organised animal and vegetable forms, however,

[1] "Die Kleberschicht des Grasendosperms als Diastase ausscheidendes Drusengewebe," 'Berichte der deutschen botan. Gesellsch.,' 1890, p. 40. Abstract in 'Botan. Centralbl.,' vol. xliii, p. 39.

some important variations were found in the disposition of the iron-holding substance, and it was further determined that in non-nucleated organisms the exceptional distribution of the chromophilous substance is co-extensive with that of the assimilated iron compounds observed. Such facts are worthy of an extended description, and I now propose to detail these and the more important observations allied to them.

Ascaris.—In the species A. mystax the spermatozoids and ova, both before and after fertilisation, manifest special features in the distribution of the iron-containing substance. When they are hardened in alcohol, the spermatozoids are comparatively easily affected by the ammonium hydrogen sulphide, the reagent, mixed with glycerine, giving in a couple of days, under the usual conditions, a reaction for iron, which usually is confined to the "nucleus," a dense homogeneous body (fig. 31); but in several instances the "membrane" also contained iron. The reaction in the latter varied in intensity, and when most marked it revealed a structure in the "membrane" like that represented in fig. 32. The iron compound observed in such a case obtained only in the rodlets constituting the "membrane." What the occurrence of assimilated iron in this situation signifies I am unable to say, except that it possibly represents an abnormal phase of a condition normal to the spermatozoid after it has penetrated the ovum. When the spermatozoid begins to penetrate the latter, its membrane frequently manifests a weak reaction for iron (fig. 29), while its cytoplasm does not give any evidence of the presence of that element; but in the changes it undergoes after reaching the interior, the "nucleus" becomes in part dissolved, and the chromatin, as shown by the iron reaction, diffuses into the cytoplasm and into the membrane, from which some of it passes into the cytoplasm of the ovum immediately adjacent to the spermatozoid. The membrane in this way becomes the most prominent part of the spermatozoid. As the transformation proceeds, the membrane also dissolves, and the iron which it contains appears to pass back again into the cytoplasm of

the spermatozoid, but what is held in the cytoplasm of the ovum apparently is retained by the latter.

These observations on the diffusion of the iron-holding substance from the "nucleus" of the spermatozoid into its cytoplasm coincide with those of van Beneden upon the changes which take place in the spermatozoid of Ascaris megalocephala after it penetrates the ovum. He found that the protoplasm of the free spermatozoids[1] manifests no affinity for staining compounds, while its capacity for absorbing and retaining all colouring matters becomes remarkable immediately after it enters the ovum. As the "nucleus" at the same time loses in part its affinity for stains, he came to the conclusion that a part of the chromatic substance (chromatin) of the "nucleus" becomes dissolved in the cellular substance (cytoplasm). O. Zacharias[2] has also pointed out that the protoplasm of the free spermatozoid, apart from its "nucleus," is absolutely unstainable, but after it penetrates the ovum it at once manifests an affinity for colouring matters. Kultschitzky,[3] referring to the reactions with staining fluids, suggests that possibly the "nucleus" gives off to the cytoplasm of the spermatozoid a portion of its chromatin, or that, in other words, not all of the chromatin of the "nucleus" is employed in the construction of the male pronucleus. I have found in my preparations that the cytoplasm and "membrane" of the spermatozoid which has penetrated the ovum, and, frequently also, that portion of the cytoplasm of the ovum in the immediate vicinity of the spermatozoid, have a slightly greater affinity for colouring matters than the cytoplasm of the free spermatozoid or of the unimpregnated ovum.

In many of his illustrations van Beneden represents that part of the spermatozoid which I have called the "membrane"

[1] "Recherches sur a maturation de l'œuf et la fécondation," 'Archives de Biologie,' vol. iv, p. 265, 1883.

[2] "Neue Untersuchungen über die Copulation der Geschlechtsprodukte und den Befruchtungsvorgänge bei Ascaris megalocephala," 'Arch. für Mikr. Anat.,' vol. xxx, p. 111, 1887.

[3] "Die Befruchtungsvorgänge bei Ascaris megalocephala," 'Arch. für Mikr. Anat.,' vol. xxxi, p. 567, 1888.

as deeply stained, and in these one finds the existence of rodlets indicated, such as those to which I have referred above; but these (les stries transversales de la queue) are more apparent in the penetrating than in the free spermatozoid. I observed only faint traces of such structures in the spermatozoid in the interior of the ovum, the rodlets apparently commencing to disappear immediately impregnation is accomplished.

The chromatin of the nucleus of the ovum gives a deep reaction for iron in whatever stage the nucleus may be found (figs. 29 and 30). The chromatin also of the "polar globules" contains iron, and I made efforts to determine the ultimate fate of this, but these were unsuccessful. It would appear, however, as if the chromatin of the extruded elements were dissolved eventually in the cytoplasm, for it is impossible to find any traces of it after a time.

Chironomus.—Balbiani,[1] who was the first to call the attention of cytologists to the structure of the nuclear elements in the "salivary" glands of the larva of Chironomus, described the nuclear filament as made up of a series of dim discs or bands, each placed transversely, and separated from its neighbour on either side by a band of clear substance, the filament possessing, however, at certain points an annular swelling, and terminating at its ends either in the polymorphous nucleolus or by an attachment to the nuclear membrane. Leydig,[2] the next observer, found each dim stria to be made up of a series of elements whose separation from each other gives a composite character to the stria. The fine lines separating the elements are, according to his observation, continued from one dim disc, through the light disc on either side of it, to the adjacent dim disc. In this way a series of exceedingly delicate longitudinal lines, in addition to the coarse transverse ones described by Balbiani, make their appearance. Leydig also believes that the substance forming the dim band

[1] "Sur la structure du noyau des cellules salivaires chez les larves de Chironomus," 'Zool. Anzeiger,' 1881, pp. 637 and 662.

[2] 'Untersuchungen zur Anatomie und Histologie der Thiere,' Bonn, 1883, p. 90.

is situated immediately under the membrane. Korschelt's views on the structure of the filament are directly opposed to those of Leydig and Balbiani. He regards the transverse striation of the filament as due to a folding of the surface membrane only, and explains the longitudinal striation observed by Leydig as caused by the action of the reagents used. In his opinion, also, the apparent differentiation of the filament is due to the differences in the reflected light.

So far as I know, no one has hitherto observed an arrangement in the nuclear filament of Chironomus similar to that described by Leydig, although Carnoy has found in the salivary gland of a Nemocere larva that the dim disc is formed of a series of longitudinally disposed rodlets, but he attributed the delicate lines observed in the clear discs to folds in the membrane of the filament.[2] The larvæ of the species of Chironomus accessible to me offer preparations less favourable for study than do those of the species C. plumosus studied by Balbiani and Leydig, yet I have been able to determine, with my methods for demonstrating the presence of assimilated iron, the correctness of Leydig's observations so far as they go. The dim discs are of different thicknesses, the thickest appearing to be five or six times the diameter of the narrowest. When the salivary gland, after being hardened in alcohol, is kept for several days in sulphuric acid alcohol, treatment with an acid ferrocyanide solution gives all these dim bands a deep blue reaction, the intensity of the reaction coming out very markedly in the thicker bands. Under the highest magnification of service in such a case (apochromatic immersion 1·5 mm. and compensation ocular 8, Zeiss), the bands of medium thickness are resolved into a series of short rodlets disposed parallel with the filament. If the filament has, in the course of preparation, been isolated from the nucleus, one may then determine that the rodlets forming one dim band are connected by excessively delicate fibrils with the rodlets

[1] Ueber die eigenthümlichen Bildung in den Zellkernen der Speicheldrüsen von Chironomus plumosus," 'Zool. Anz.,' vol. vii, pp. 189, 221, 241, 1884.

[2] 'Biologie Cellulaire,' p. 232.

forming the two adjacent bands. The fibrils, or what are in appearance such structures, have so little iron in them that frequently in a large part of an isolated filament their blue reaction may not be sufficiently deep to betray their presence, but the chances of observing them may be increased by staining such preparations carefully with safranin. Probably the expression fibril is not a correct one to apply to these appearances, for they may be the optical sections of the partition walls of compartments, the extreme ends of which would in that case be formed by the dim bands. What appears to support the latter view is the fact that in some of the thickest dim bands the Prussian blue reaction reveals the presence of a single row of vesicles extending from one end of the band to the other, the vesicles sometimes having an elongated form parallel with the filament. It seemed to me that these were the initial stages in the division of one dim band into two, that the thinner bands represent those most recently formed, and that, therefore, the vesicular mode of formation would result in the production of a series of compartments the thin walls of which, in the clear bands, would appear as fibrils. The structures observed are, however, so exceedingly minute that it is impossible to determine definitely anything on this point.

The iron-holding substance in the filament is, therefore, disposed in the rodlets of the dim band and in the fibril-like elements connecting the rodlets of one dim disc with those of its neighbours. The only exception to this statement may be made in regard to the structure of the swellings which are sometimes found on the course of a filament (fig. 50). In this case the dim discs are replaced by an iron-holding reticulum disposed in the interior of the swollen portion of the filament. A comparison of this portion with the adjacent portions of the filament appears to indicate how the reticulum has arisen and what its relations are. The iron-holding bands on either side are less regular in their disposition than elsewhere, and the fibril-like structures arising from them appear to be directly connected with the iron-holding substance of the reticulum referred to. The swollen portion of the filament

varies in its size and shape, but most frequently it has the appearance represented in the figure.

I have never observed the annular swellings described by Balbiani as present in the filament in C. plumosus, but I take it that the swollen portions here described are the representatives of such structures. Nor have I ever determined that the filament ends by attachment to the nuclear membrane, or to the amœbiform nucleolus, through which it may pass several times in its course. The nucleolus varies not only in form and size but also in composition. It may be homogeneous, but more frequently the central portion contains vacuoles and granules and stains more deeply with eosin or safranin, while the peripheral non-granular portion may possess no staining capacity whatever. In many preparations made from alcohol material and stained with eosin, the nucleolar body alone is stained, and this is particularly the case when the preparation has been treated with acid alcohol and the acid ferrocyanide mixture to demonstrate the iron present. The nucleolar substance, apart from its granules, contains iron, but the iron present is very small in amount compared with that observed in the filament, for, when the latter gives an intensely deep blue reaction, the colour given the nucleolus is a very pale blue, and when the nuclei are kept for a week mounted in the glycerine and sulphide mixture in the warm oven, the isolated nucleoli develop only a greenish colour, portions of the filaments, on the other hand, giving in the same preparations a marked dark-green reaction. Unlike the differences in staining exhibited after treatment with eosin, the faint or light blue reaction is uniform throughout the nucleolar substance.

The dim bands with the excessively fine fibrils in the filament are formed of chromatin, as shown by treatment with the staining reagents, when the preparations have been properly hardened. There is, however, a difference between this chromatin and that of the ordinary animal cell in that while acid methyl-green colours the former it leaves unaffected the nucleoli and the swollen portions of the filament, which stain deeply with hæmatoxylin and carmine.

Balbiani[1] concluded from such results that chromatin (substance chromatique) is present, not only in the dim discs, but also in the annular swellings and the nucleoli. According to Flemming,[2] safranin colours all these elements, but stains the nucleoli very strongly. Flemming's observation is correct only for preparations made with the chrom-osmio-acetic reagent; but when the nuclei have been fixed with alcohol, or with corrosive sublimate, treatment with acid alcohol for two or three days affects the filament in such a way that its discs and their excessively fine fibrils absorb and retain the safranin to a very marked extent, while the nucleolus remains unstained, and the swollen portions of the filament are faintly coloured. It is possible to obtain in such preparations both the safranin and the Prussian blue reactions, and then, with the exception of the faint blue in the nucleoli, both effects are co-extensive and of equal intensity. The marked difference between the substance of the discs and that of the nucleoli is thus shown, but it may be brought out in a more brilliant way by staining Prussian blue preparations with eosin, which then affects the nucleolus only.

The nucleolus thus resembles the similarly named structure obtaining in the nuclei of Vertebrates, but it differs from this in that it is amœboid in form, and does not possess, in any case, a chromatin envelope. The presence of granules and vacuoles, moreover, appears to indicate that it is physically active, which cannot be postulated of the vast majority of the nucleoli of Vertebrate cells.

Whatever effects may be obtained by treating the nuclei with various staining reagents, but one results in the cytoplasm of the secreting portions of the salivary gland in Chironomus. Acid methyl-green in the fresh preparations, and hæmatoxylin and safranin in the hardened glands, demonstrate very clearly that there is a stainable substance, in many respects like chromatin, uniformly distributed through the cytoplasm; that it is chromatin would appear from the fact that the cytoplasm

[1] Loc. cit.

[2] 'Zellsubstanz, Kern- und Zelltheilung,' pp. 112, 113.

holds an assimilated iron compound, for if small fragments of cells, hardened in alcohol, be subjected to the action of the warm glycerine and sulphide mixture for a week or more, they will manifest a dark-green reaction which, when the mixture is washed away and replaced by an acid ferrocyanide solution, is converted into that of Prussian blue. One may more readily obtain the demonstration of the iron in these cells by allowing sulphuric acid alcohol to act on the hardened gland for two days, when the cytoplasm of the secreting cells and the substance of the thread (silk?) in the lumen give evidence of the presence of this element. Whether the iron thus demonstrated in the substance of the thread belongs to the latter, or is derived by diffusion from the cytoplasm of the secreting cells during treatment with the acid alcohol, I am unable to say, since my experiments made to determine this question, by the use of the glycerine and sulphide mixture on isolated bits of the threads, turned out to be failures.[1] The substance forming the threads manifests a strong affinity for dyes, and should it eventually be ascertained that the iron demonstrated in it, after treatment with acid alcohol, is part of a "masked" compound contained in it, the facts will then all indicate that the iron-containing substance in the cytoplasm is the antecedent of at least a portion of the substance of the thread in the lumen, and one will have then also a parallel of what was pointed out as obtaining in the pancreas and other ferment-secreting cells in Vertebrates.

Protozoa.—I have selected the genera Stentor, Epistylis, Vorticella, and Paramœcium for specially illustrating the distribution of the assimilated iron in unicellular animals. A very large number of other forms were used to confirm the results which a study of the named organisms gave, but owing

[1] Gilson (loc. cit.) has referred to the fact "that the silk of certain insects seems to possess a stronger affinity for this metal (iron) than nuclein itself." I have observed this peculiarity, but the iron absorbed is at once demonstrated on the application of any form of ammonium sulphide, a fact which shows that the iron so revealed does not enter into a "masked" condition, and ought not to be confused with that of "masked" compounds.

to the difficulty experienced in getting examples of such forms in the numbers required, it was impossible to make a fully satisfactory, systematic investigation of their iron-holding character. On the other hand, examples of the genera named could be obtained at all times in abundance, and I regard the opportunities thus presented as compensating in some measure for the limited range of genera studied.

One of the difficulties encountered in attempting to study the distribution of iron-compounds in Protoza is the fact that many of the motile forms, and some also of those which are sessile or attached, have in their cytoplasm inorganic compounds of iron, in great part, if not wholly, derived from the food matters ingested, and when such organisms, after being hardened in alcohol, are treated with the glycerine and sulphide mixture, they give at once a deep reaction for iron which, in many cases, obscures other details in the cytoplasm and nucleus. When, moreover, attempts are made with acid alcohols, and especially Bunge's fluid, to remove the inorganic iron, the conditions under which the experiments are made enable the reagent to liberate the "masked" iron at the same time, in which case the liberated portion becomes indistinguishable from that present previously in an inorganic form. To avoid such difficulties it is necessary to select forms in which the amount of inorganic iron is small or infinitesimal, and by determining the amount of the reaction obtained during the first ten minutes after the application of the glycerine and sulphide mixture, one may thus prevent confusion arising from the study of results obtained by the more prolonged application of the reagent. Such forms may be found in the genera above named, and one may, by attention to the character of the medium of the organisms, without any difficulty secure such examples as offer the most favourable conditions for investigating the distribution in them of the assimilated iron. The specimens of Epistylis, for example, which were used by me for this purpose, were obtained from a colonial form attached to the sides and limbs of the common crayfish, and their cytoplasm gave no immediate reaction for iron. Examples of

Stentor and Paramœcium, in sufficiently large numbers, and all but completely free from inorganic iron compounds, were readily obtained. The cytoplasm in Vorticella, on the other hand, usually contains such compounds, but these are very often in the form of granules situated in vacuoles, or at the periphery of the same, a disposition of the compounds which gives every facility for studying the distribution of the assimilated iron.

In the examples of Epistylis there were, as stated, no inorganic compounds of iron, at least none were demonstrable in the glycerine and sulphide mixture within the first hour after the application of the reagent, but on the third and fourth day both cytoplasm and nucleus gave a marked reaction for iron. The latter was, of course, most prominent in the nucleus, in which was revealed, by the dark-green colour, in some examples a granular structure, in others a fibrillar arrangement. The reaction of the cytoplasm was a diffuse one, with here and there large granules in which it had developed more markedly. The membrane and stalk were, in these cases, free from iron. All these points were more readily observed in preparations treated with sulphuric acid alcohol or with Prange's fluid for twenty-four hours (fig. 28).

In Vorticella a similar distribution of the assimilated iron was observed in both cytoplasm and nucleus, and a diffuse reaction for iron was also obtained in the central or axial portion of the stalk, after the preparation had been kept in the warm glycerine and sulphide mixture for several days. The reactions are represented in fig. 27, drawn from a preparation which contained inorganic iron compounds disposed in vacuoles. In this the central portion of the stalk is shown to be continued into a funnel-shaped organ at the base, which also contains "masked" iron. I was unable to determine how this organ was connected with the cytoplasm. I found no difficulty in obtaining the complete reaction in all the parts at the end of a five days' application of the warm glycerine and sulphide reagent.

Examples of Stentor polymorphus, free from inorganic

iron compounds, were, after being hardened in alcohol and after treatment with ammonium sulphide, isolated from those more or less impregnated with iron salts, the large size of the organisms enabling one to do this readily. One of such, after treatment for fourteen days with the warm glycerine and sulphide mixture, is represented in fig. 25. In this no distinct reaction was obtained during the first two days, definitely showing that no inorganic iron was present. In the interior of the spherical elements constituting the nucleus there appeared eventually a diffuse iron reaction, as well as one localised in granules, and the cytoplasm gave a diffuse reaction like that given by the cytoplasm in Epistylis and Vorticella. I do not think that in this case the reaction had developed to the fullest extent of which it was capable, for I found other examples in which the nuclear and cytoplasmic elements gave a more intense one; but it is usually difficult in such large cells to obtain the best effects of the reagent, since in two weeks' time it is apt to undergo decomposition, when the development of the iron reaction ceases. In order to ascertain how abundant the assimilated iron is, I employed acid alcohols to liberate it, and, after the removal of the acid, treated the preparation with ammonium sulphide. Sulphuric acid alcohol is the best reagent for the purpose, since with it there is less iron diffused from the parts in which it is liberated; but, in order to get the most exact results, the examples of Stentor used should be free from inorganic iron compounds, a point of which one may be certain by putting the hardened examples in ammonium sulphide for a few minutes, when, if they pass this test, they may be washed in alcohol to remove all traces of the reagent and placed in the acid alcohol for one or two days. I have represented in fig. 26 an example of S. polymorphus, in the wall of the funnel-shaped œsophagus of which was found the only inorganic iron compound present, and in this, after it had been treated as described, the ribbon-like nucleus appeared intensely greenish-black, while the cytoplasm gave a deeper reaction than was obtained in any specimen simply by prolonged treatment of it with the warm

glycerine and sulphide mixture. In examples absolutely free from inorganic iron compounds the reaction in the cytoplasm and nucleus was as marked as that represented in the figure. The method is, of course, open to the objection that it may permit a diffusion of the liberated iron from the nucleus to the cytoplasm, but that the latter contains assimilated iron is shown by prolonged treatment with the warm glycerine and sulphide reagent.

In examples of different species of Paramœcium, the cytoplasm, which gave no reaction for inorganic iron, manifested with the warm glycerine and sulphide reagent after ten days a reaction as distinct as that obtained under similar conditions in the cytoplasm of Stentor, Vorticella, and Epistylis. These organisms were the only ones in which the micro-nucleus was revealed by the iron reaction, and the latter appeared to me to develop more slowly than that in the macro-nucleus; but the explanation for this may be that the large quantity of chromatin in the latter renders a reaction of any degree of intensity obtaining in it much more prominent than a reaction of a similar intensity would appear in the micro-nucleus. In both the reaction was almost wholly confined to the granules and fibrillar elements.

All the forms of Protozoa studied illustrated the fact so prominently indicated in the organisms referred to above, that an assimilated compound of iron is a constant element in their cytoplasm. It is probable that this compound belongs to the chromatin class, for the cytoplasm in Protozoan organisms generally stains much more readily, and holds the dyes more tenaciously, than the cytoplasm in higher organisms does. In support of this may be urged other facts. I pointed out, when dealing with the relations of assimilated iron compounds to the ferment-forming cells in Vertebrates, that the substance which elaborates the ferment, or out of which it is prepared, contains iron and acts towards staining reagents like chromatin. Digestion in Protozoa is, in all probability, effected by ferments derived, as in higher forms, from the cytoplasm, and it is only reasonable to suppose that

the antecedent of the ferments is, in this class also, an iron-holding chromatin.[1]

Euglena viridis is a form whose position, whether as a vegetable or as an animal organism, has not by any means been definitely determined, but the distribution of assimilated iron in its interior appears to indicate that if it does not belong to the animal kingdom, its physiological processes possibly resemble those of the Protozoan cell, and it is for this reason that I deal with it in this place. Examples of this organism free from inorganic compounds of iron may be obtained readily, and when hardened in alcohol, they may be subjected to the action of the glycerine and sulphide mixture for twenty-four hours, without manifesting a reaction for iron, but when the application is extended for three days or longer, a reaction for iron is obtained in the nucleus and cytoplasm. The chromatin network is usually so affected by the reagent that its nodal points only manifest the reaction, while the nucleolus exhibits a less intense dark-green colour. The cytoplasmic trabeculæ separating the "amylaceous" corpuscles from each other develop a dark-green reaction, which is found to be most intense at the nodal points. All these features are more clearly seen in specimens which have been hardened in alcohol, then treated for two days with sulphuric acid alcohol, and finally, after being acted on with the acid ferrocyanide mixture to produce the Prussian blue reaction, mounted in balsam (fig. 49). In these preparations the iron revealed in the cytoplasm is most abundant in its nodal points, which, with the reticulum of the nucleus, are thereby rendered most prominent. The nucleolus, separated from the other elements by a clear zone, in which the light blue observed is derived from the nuclear elements above and below the focal plane, gives a less intense reaction than one of the much smaller nodal points of the nuclear network. If the preparation has also been stained with eosin the nucleolus alone appears to be

[1] The ferment or ferments, according to M. Greenwood ('Journal of Physiology,' vol. viii, 1887, p. 263), pass into the fluid surrounding the ingested matter.

markedly affected by it, exhibiting an ochre-red colour so characteristic of the nucleoli in the hepatic cells of Necturus after similar treatment. Safranin leaves the nucleolus unaffected, but colours deeply the chromatin network and the iron-holding portions of the cytoplasm. When, however, the organism has been hardened in picric acid, the nucleolus exhibits no affinity for eosin, while it colours as deeply as the chromatin network does with hæmatoxylin and picro-carmine. From this it would appear as if the nucleolus were intermediate in composition between the nucleolus of higher animal cells and the chromatin of the nuclear reticulum.

The occurrence of assimilated iron in the cytoplasm of Euglena viridis, it it is not chemically associated with the chlorophyll present, appears to indicate that the organism is closely related to the Protozoa, in common with which it has other characters.[1] If the view, that the assimilated iron in the cytoplasm of Protozoa is part of the antecedents of the zymogenic compounds of these organisms, is correct, it would explain the phenomenon in Euglena in which the presence of a short digestive "tract" also postulates, to a certain extent, the occurrence of processes of nutrition belonging to the animal type.

Fungi.—The presence of nuclei has not yet been demonstrated in a large number of the Fungi, nor has the occurrence of a substance similar to the chromatin of other organisms been determined with any degree of certainty, except in a few forms; and, therefore, the question of the occurrence and distribution of assimilated compounds of iron in the cells of this class is not quite as easy of solution as that dealing with the

[1] G. Klebs, who has given special attention to the Euglenaceæ ("Organization einiger Flagellaten-Gruppen und ihre Beziehungen zu Algen und Infusorien," 'Untersuch. aus dem Bot. Inst. zu Tübingen,' 1881–85), is of the opinion that this group should be classed amongst the Protozoa. Khawkine ("Recherches biologiques sur l'Astasia ocellata, n.s., et l'Euglena viridis. Seconde Partie, L'Euglena viridis." 'Ann. des Sciences Nat., Zoologie,' Serie 7, vol. i, 1886, p. 319) came to the conclusion, as a result of experiments, that Euglena takes in organic compounds in the dark, but in daylight assimilates only inorganic compounds.

presence of these compounds in higher organisms. I have, however, endeavoured to solve it by the investigation of a few widely different forms, and the results now to be described show the presence of "masked" iron compounds similar to those found in all the higher organisms. These forms comprise: Saccharomyces cerevisiæ, S. Ludwigii, Hyphelia terrestris Fries, a leucosporous Agaricine, Cystopus candidus, and Aspergillus glaucus.

The question of the occurrence of a nucleus in Saccharomyces bears upon that relating to the presence of iron-containing chromatin-like substances in this genus; and, consequently, it is necessary to give an account of the various observations that have been made on this subject.

The earlier botanists, Nägeli[1] and Schleiden,[2] claimed that they had found a nucleus in the yeast-cell, and the later observers, Schmitz,[3] Strasburger,[4] Zalewski,[5] and Zacharias,[6] have maintained that it exists, while Zimmerman[7] speaks reservedly on the question. Raum[8] found in yeast-cells which had been fixed on the cover-glass by heat or by solutions of corrosive sublimate, and stained, first with warm methylene-blue and then with bismarck brown, black spherical granules, varying in number from one to fifteen, in a more or less brown-tinted protoplasm. These were usually arranged in the

1 'Zeit. für wiss. Botanik,' vol. i, p. 45. Reference in Raum's paper.

2 'Grundzüge der wiss. Botanik,' 1849, p. 207. Referred to by Raum.

3 "Untersuchungen über den Zellkern der Thallophyten," 'Sitzungsber. der Niederrhein, Gesell. für Natur- und Heilkunde zu Bonn,' Sitzung. am 4 Aug., 1879.

4 'Das Botanische Practicum,' p. 339, 1887.

5 "On Spore Formation in Yeast Cells," 'Transactions of the Scientific Academy of Cracow' (Polish), 1886. Abstract in 'Bot. Centralbl.,' vol. xxv, p. 1.

6 "Beiträge zur Kenntniss des Zellkerns und der Sexualzellen," 'Bot. Zeitung,' 1887, Nos. 18–24.

7 "Die Morphologie und Physiologie der Pflanzenzelle," Breslau, 1887, p. 25. I have not had access to this publication, and my attention was first called to it by a reference made by Raum.

8 "Zur Morphologie und Physiologie der Sprosspilze," 'Zeit. für Hygiene,' 1891, vol. x, p. 1.

form of a circle or of a segment of a circle at either pole of the oval cell, and there was no relation between their size and that of the cell containing them, although they appeared to have some connection with the budding process, since he observed them undergoing transference to the protoplasm of the bud. What the nature of these granules is Raum does not say, but the results of his experiments would seem to indicate that they are not formed of nuclein, for on submitting the yeast-cells to digestion with an artificial gastric fluid at a temperature of 40° C. for one or two days, and afterwards on washing with ether and alcohol, every trace of the granules had vanished. Nuclein is undoubtedly present in yeast-cells, and Raum prepared some of it from this source, which he mounted in egg-albumen on a cover-glass, and stained, first with methylene blue and afterwards with bismarck brown, when he found that the nuclein particles took a brownish stain while the albumen appeared light yellow, a reaction in marked contrast with that obtained in the granules of the hardened yeast-cells after the employment of the same staining methods. Raum appears to be doubtful concerning the existence of anything resembling a nucleus in the yeast-cell.

The more recent observers who claim to have found a nucleus in the yeast-cell are Möller and Janssens. The former[1] found in older yeast-cells a spherical corpuscle which he regards as a nucleus, but without a membrane or nucleolus. This changes its shape readily, and therefore its position in the cell varies. Owing to this property, it is capable of assuming a thread-like form when budding occurs, a portion of it being thus enabled to pass into the protoplasm of the bud through the narrow tube which connects the mother and daughter elements. The part in the latter eventually breaks off, and both portions become spherical. Janssens,[2] who used

[1] "Ueber den Zellkern und die Sporen der Hefe," 'Centralbl. für Bakt. und Parasitenkunde,' vol. xii, 1892, p. 537; also "Weitere Mittheilungen über den Zellkern und die Sprosse der Hefe," ibid., 1893, vol. xiv, p. 358.

[2] "Beiträge zu der Frage über den Kern der Hefezelle," 'Centralbl. für Bakt. und Parasitenkunde,' vol. xiii, 1893, p. 639.

in his investigations the species S. cerevisiæ, S. Ludwigii, and S. Pastorianus, states that he found in the two former a nucleus provided with a membrane and a nucleolus, the latter spherical and homogeneous and of a diameter one third that of the nucleus. The remaining portion of each cell is occupied by a cytoplasmic network with fine meshes, whose nodal points readily absorb colouring matters, and, in the opinion of Janssens, constitute the granules of Raum. He claims to have observed mitotic stages of the nucleus, which obtain when budding commences and when spore formation occurs.

Two observers only, Brücke[1] and Krasser,[2] have denied the existence of a nucleus in the yeast-cell. Krasser in his later publication asserts that the body described by Möller as a nucleus is not such an organ, and he found, after employing Möller's methods on beer yeast-cells, that the latter possessed no body like the one described by that observer. He further observed that the bodies described by Möller as nuclei, after being submitted to digestion with artificial gastric juice, gave no evidence of the presence of nuclein. The occurrence of the latter substance in yeast-cells, which is readily demonstrable in a macro-chemical way, Krasser attempted to show micro-chemically, and, after many failures, succeeded in finding it in a few specimens in the form of granules at the side of the body regarded by Möller as a nucleus.

I have followed the methods of hardening and staining adopted by Möller, for the purpose of ascertaining the nature of the body considered by him to be a nucleus, and have compared the results thus obtained with those found in yeast-cells after hardening the latter in saturated solutions of corrosive sublimate and staining them with hæmatoxylin and eosin. I have also used Flemming's fluid for hardening, and stained preparations so made with safranin. Möller's methods certainly do reveal, now and then, a structure like that which

[1] "Die Elementarorganismen," 'Sitzungsber. der K. Akad. d. Wiss. zu Wien, Math.-Nat. Classe,' 1861, vol. xliv, Abth. 2.

[2] "Ueber das angebliche Vorkommen eines Zellkerns in den Hefezellen," 'Oesterreich. Bot. Zeits.,' 1885, No. 11; also "Ueber den Zellkern der Hefe," ibid., 1893, p. 14.

he took to be a nucleus, but this body, when hardened with corrosive sublimate, stains with eosin but not with hæmatoxylin, while after fixation with Flemming's fluid it appears to have no particular affinity for any dye. On the other hand, in S. Ludwigii, as it usually develops in the sap of the iron-wood tree (Ostrya virginica), there is in the great majority of cells a corpuscle which corresponds with the "nucleus" of Möller. This structure is round, homogeneous, and in diameter sometimes more, sometimes less, than half the length of the shorter axis of the cell, in the centre of which it is usually placed, and after being hardened with corrosive sublimate it exhibits a special affinity for eosin, but none for hæmatoxylin, while it acts like the cytoplasm towards safranin. In preparations made with Flemming's fluid the results were practically the same, and therefore not indicating on the part of the body in question the possession of a substance in all points like chromatin.

A substance like chromatin appears to be distributed through the cytoplasm. In S. cerevisiæ, after being hardened with corrosive sublimate, the cytoplasm takes, when treated with hæmatoxylin (Delafield's and Ehrlich's), a blue-violet tinge. With favourable illumination and apochromatic objectives, the stain is found to be localised in the trabeculæ of the cytoplasmic network, and, where the vesicular character of the cytoplasm appears pronounced, all the cytoplasm, except the contents of the vesicles, is coloured. In some of the cells granules were observed with a stain slightly deeper than that of the cytoplasm, and similar elements were found in cells hardened with Flemming's fluid and stained with safranin. These, possibly, are those described by Raum. In S. Ludwigii the cell is usually very much larger, and the structure and staining reactions are, therefore, much more distinct. In this form, when hardened with corrosive sublimate and stained with hæmatoxylin, the vesicular structure of the cytoplasm comes out quite markedly through its blue-violet stain, which also is found now and then to characterise prominently granules in the cytoplasm between the vesicles. The granules of

Raum are, however, much more common elements than these, and are to all appearances quite different structures, as is apparent in ordinary cover-glass preparations made after Raum's methods. The larger examples of the granules of Raum seem to be less abundant in corrosive sublimate preparations stained with hæmatoxylin and eosin.

From these results I am inclined to regard the existence of a nucleus in the yeast-cell, in its usual condition, as extremely doubtful, and, on the other hand, to support Krasser's contention that nuclein is disseminated through the cytoplasm. Whether, in other stages, as, for example, those in which spore formation occurs, there is a nucleus I cannot say, but there appears in the ordinary stages of the organism to be nothing which may be looked upon as a specialised chromatin-holding structure.

These conclusions are, on the whole, confirmed by the results of experiments made to determine the distribution of assimilated compounds of iron in these organisms. When specimens of S. cerevisiæ, hardened in alcohol, are subjected to the action of the glycerine and sulphide mixture at a temperature of 60° C. for several days, their cytoplasm acquires a greenish tint. Sometimes, however, the latter reaction may not appear except in a few granules scattered through the cytoplasm (fig. 4). On account of the small size of the cells and of the alteration produced in them by the reagent, one cannot definitely determine whether the granules correspond to those described by Raum. When the cells have been subjected to the action of sulphuric acid alcohol, the subsequent application of an acid ferrocyanide solution gives their cytoplasm a faint blue colour, which is more distinct and deeper when the light transmitted passes through several cells in succession. Blue granules are sometimes observed in such preparations.

It is in specimens of S. Ludwigii that one obtains the clearest evidence of the occurrence of an assimilated iron compound. In these, after being hardened in alcohol, the glycerine and sulphide mixture eventually gives results like

those represented in fig. 5. The differences observed appear to depend on the cytoplasmic structure in the specimen examined. When there are a few large vesicles in the cell, the iron-holding substance seems to be, in great part, at their peripheries. This disposition also obtains in the buds. The remaining portion of the cytoplasm in each element is very slightly coloured greenish, but whether that is due to ferrous sulphide is uncertain. When, on the other hand, the cells are markedly vesiculated, the glycerine and sulphide mixture gives the cytoplasm between the vesicles a distinct reaction for iron. In the majority of such cells there are one or more large spherical elements, which, in the glycerine and sulphide mixture, after the third or fourth day appear dark green, much more so than does the surrounding cytoplasm. They are homogeneous, manifesting a uniform reaction throughout their substance, and their position is, if not in the centre of the cell, at least in that neighbourhood; but smaller granules of the same character may be more remotely situated. From their position, size, and shape, they would appear to be the bodies which, in preparations made with corrosive sublimate, hæmatoxylin, and eosin, stain exclusively with the latter reagent. In cells which are treated with acid alcohol, then with an acid ferrocyanide solution, and finally, after being stained with eosin, mounted in balsam, similar bodies are given a violet tint, while the cytoplasm is coloured bluish, the violet being undoubtedly due to a combination of the Prussian blue colour with the eosin stain. As the granules of Raum are not specially selected by eosin, it would appear that the iron-containing body observed does not belong to that class.

It is thus seen that in S. cerevisiæ the assimilated iron is, like the substance which absorbs hæmatoxylin, distributed through the cytoplasm and sometimes also in the latter in the form of granules, but in S. Ludwigii it may be chiefly found at the periphery of each large vesicle when only a few vesicles are present, while in those cells in which the whole of the cytoplasm is vesiculated, the latter gives a uniform reaction for iron corresponding in its depth with that given by hæma-

toxylin. Further, there is a substance which constitutes corpuscles of a nucleolar character in cells of this form, which stains with eosin and gives a marked reaction for iron, but differing from the chromatin substance in remaining unstained after treatment with hæmatoxylin. There is no nucleus, although such an organ may occur in other stages, especially in S. Ludwigii.[1]

When the mycelial threads and hyphæ of Hyphelia terrestris, Fries, are hardened in alcohol and stained with hæmatoxylin, the cytoplasm generally is coloured, but it is specially affected by the stain in the terminal portions of the hyphæ on which the elements of fructification are developing. One can find also, in such preparations, deeply-stained granules scattered in the cytoplasm of the hyphæ, and at times also a vesicular cavity and a membrane enclosing these granules, which then simulate nucleoli. Sometimes such structures strongly resemble nuclei, and mitotic conditions are suggested by the presence of pairs of rows of deeply-coloured granules placed opposite, and at a very short distance from, each other. In the fully-formed fructification these vesicular cavities and their deeply-stained granules may be most readily seen. Whether such structures are nuclei in the proper sense of the term it is difficult to say, but if they are, they contain only a small portion of what may be considered as the chromatin, which is diffused in the cytoplasm of the mycelial threads in the younger stages, but appears to be transferred to the hyphæ when the fructification of the latter commences. When the latter stage is fully attained the mycelia and lower portions of the hyphæ are found to have little or no cytoplasm and to stain very feebly, a result quite different from that obtained in the fructification.

The distribution of the "masked" iron in this form is found to coincide very closely with the distribution of the stainable substance. In the simplest form of the hypha, the glycerine

[1] Ludwig ('Lehrbuch der niederen Kryptogamen,' 1892, p. 201) appears to regard S. Ludwigii as merely a stage in the development of Endomyces Magnusii.

and sulphide mixture gives in twenty-four hours a reaction like that represented in fig. 13 *a*, while in the slightly more developed structure the reaction is deeper with large dark-green granules (fig. 13 *b*). A similar result is obtained in the hyphæ which terminate in two, three, or more pear-shaped outgrowths (fig. 12). In the hyphæ below the fructification the cytoplasm is of a vesicular character, the walls of the vesicles being formed of an iron-holding substance, and as the terminal element develops, the vesicular character becomes less marked and the iron reaction less distinct, so that, finally, no iron may be found in this part of the filament. At the same time the granules in the fructification become more numerous, larger, and manifest a deep reaction for iron (fig 11). These granules are then found to be situated in small vesicles very much like the vesicles which, in hæmatoxylin preparations, resemble nuclei. The granules revealed by the iron reaction are the same as those indicated by the hæmatoxylin stain. This is also true of the granules in the younger hyphæ. The cytoplasm of the mycelial threads is, at this stage, free from "masked" compounds of iron, but in the earliest stages the mycelial threads give at once, on the application of the glycerine and sulphide mixture, a slight reaction for iron, which, however, becomes deeper at the end of twenty-four hours if heat be applied, this indicating the presence of "masked" iron. Granules in the cytoplasm along the course of the threads give a marked reaction for the metal like that manifested in the hyphæ. It is probable that the absence of iron in the later stages of the threads may be due to the transference of the iron-holding compound to the hyphæ.

The question concerning the occurrence of nuclei in the Hymenomycetes has been dealt with by Strasburger,[1] Rosenvinge,[2] and Wager.[3] The two former describe them as obtain-

[1] 'Das Botanische Practicum,' pp. 301 and 433, 1887.

[2] "Sur les noyaux des hyménomycètes," 'Annales des Sciences Nat., Bot.,' 1886, Serie 7, vol. iii, p. 75.

[3] "On the Nuclei of the Hymenomycetes," 'Annals of Botany,' 1892, vol. vi, p. 146.

ing in the hyphæ, in the basidia, and in the spores of the various species, in the form of small elements which are brought into view only when alcoholic material is acted on by very dilute solutions of hæmatoxylin. Their number in a hypha varies, but in each basidium there is at first only one, which, when the sterigmata are being formed, divides, the daughter nuclei undergoing division also, sometimes a second time, each of the four or eight thus resulting passing through the tubes of the sterigmata into the spores at the end of the latter. When the spores are mature they thus contain, according to the species, one or two very minute nuclei, while the basidia at this stage contain none. Wager also found nuclei in the basidia, but maintains that the spores do not contain any until after the formation of the thick spore-membrane.

It is an easy matter to demonstrate in the hyphæ and sometimes in the basidia and in the mature spores of leucosporous Hymenomycetes,[1] the structures regarded by Strasburger and Rosenvinge as nuclei, but, as was the case in Hyphelia terrestris, such elements contain only a small portion of the chromophilous substance, for when preparations are made, as recommended by Strasburger, with very dilute solutions of hæmatoxylin, the cytoplasm also stains though not quite so deeply as the minute nuclei, especially in young hyphæ. This and other staining reactions indicate that chromatin is dissolved in the cytoplasm, a conclusion borne out by the results of experiments with the glycerine and sulphide mixture and with acid alcohols, in which case the hyphal elements of a very young stage of growth give a reaction for iron diffused throughout the cytoplasm, but when the spores are formed the hyphal cells and their shrunken nuclei rarely give a reaction for iron. At this stage also, in sections of the lamellæ, a reaction for iron is obtained in the hymenium and in the spores, while the hyphal elements of the "trama" appear free from the metal. If the spores and the basidia are teased out and mounted in the

[1] The pigment in the spores of the other divisions of the Hymenomycetes greatly obscure the reaction obtained with the glycerine and sulphide mixture.

glycerine and sulphide mixture, the application of heat to the preparation for a week will bring out appearances in the isolated elements like those represented in fig. 10. The most prominent feature in these is that the cytoplasm in both classes of structures contains "masked" iron. When the bodies regarded by Strasburger and Rosenvinge as nuclei were observed, they manifested a slightly deeper reaction for iron than the cytoplasm generally, but no structure was detected in them and they appeared as large granules rather than nuclei. The most marked reaction for iron was obtained in the spores in which a cytoplasmic reticulum was thus demonstrated. When, however, the spores are provided with a thick membrane, a reaction with the glycerine and sulphide mixture does not appear, but is obtained after the use of acid alcohols. As a rule, the reaction is uniform throughout the cytoplasm of the basidia. There are, however, constituents of the hymenium occasionally observed in which no iron was found. They possessed no sterigmata or spores, and from their association with the basidia I was inclined to regard them as paraphyses, but from the comparative scarcity of such elements free from, or poor in iron, they can scarcely be looked upon as belonging necessarily to that class, which in stained preparations is abundantly represented. The subhymenial cells also give a faint reaction for iron.

It thus appears that in the leucosporous Hymenomycetes the cytoplasm of the hyphæ in the early stages of the fungus contains iron, which is also present in the minute "nuclei," and that in later stages this cytoplasm gives a faint reaction or none at all for iron, while the cytoplasm of the basidia and spores contains enough "masked" iron to give a marked reaction. This distribution of the iron corresponds with the distribution of the stainable substance, and it may, therefore, be fairly concluded that the chromatin is here also iron-holding.

In my earlier communication reference was made to the occurrence of an iron-containing substance in the gonidia of Cystopus candidus, and I stated that the iron compound

was found to be localised in spherical elements of 1·6μ diameter, corresponding to the nuclei of the zoogonidia. I have, since that date, investigated the cytological character of this organism, and have found that though there are, as Fisch,[1] Wager,[2] and others have observed, nuclei in the mycelia and in the gonidia, the whole of the protoplasm, except in the mature gonidia, is chromophilous, that is, it contains chromatin. The nuclei are, indeed, of the more regular form in the mature gonidia, but in the mycelia amongst the cells of the host (Capsella bursa-pastoris) they are chiefly, if not wholly, small masses of chromatin, like those forming the "nucleoli" in the abjointing gonidia. I have not succeeded in finding the mitotic phase either in the mycelia or in the developing gonidia, although I have carefully looked for such in a large number of preparations.

The disposition of the assimilated iron corresponds closely with the distribution of the chromophilous substance in this form. The cytoplasm of the haustoria and of the mycelia gave a marked reaction for iron in all the methods of demonstration.[3] The mycelial membrane gave no evidence of the presence of the element. The small masses of chromatin were found to be rich in organic iron. In the terminal enlarged, sometimes club-shaped, sometimes truncated, portion of each hypha the iron was found to be in a localised as well as in a diffuse form. The "nucleoli" gave abundant evidence of its presence, these structures thus appearing in marked contrast with the remaining portions of the nucleus, which contain relatively less iron than the surrounding cytoplasm in this stage. In the subsequent development of the abjointed gonidia, the nuclei appear to take up from the cytoplasm all, or nearly

[1] "Ueber das Verhalten der Zellkernein fusionirenden Pilzzellen," 'Versammlung deutscher Naturforscher und Aerzte in Strassburg,' 1885. This paper I have not seen, and the only references to it that I can find are those made by Wager and Dangeard ('Comptes Rendus,' cxi, 1890, p. 382).

[2] "Observations on the Structure of Cystopus candidus," 'Rep. Brit. Ass. for the Adv. of Science,' 1892, p. 777.

[3] The material was hardened in alcohol, which was renewed until every trace of chlorophyll was removed from the tissues.

all, of the iron-holding substance, and with this the character of the nuclei seems to change. The "nucleoli," first of all, are converted into fine granules distributed through the nuclear cavity, and, finally, in the mature gonidia the nuclei appear, in the glycerine and sulphide preparations, to be simply more or less homogeneous masses of iron-holding substance, while the cytoplasm does not contain a trace of the metal (fig. 6 *f*).

In Aspergillus glaucus the cytoplasm of the young mycelia and the gonidiophores, especially their globular ends, absorbs staining matters readily, but it contains also, scattered through it, granules of a nucleolar character, which, in very dilute solutions of hæmatoxylin, applied for twenty-four hours or more, stain deeply. The cytoplasm of the sterigmata and of the immature gonidia is similarly affected. In the mature gonidia hæmatoxylin selects large granules which are distributed through the cytoplasm. In what appear to be old mycelial threads, the cytoplasm is stained with difficulty, while the membrane may be deeply coloured. These results correspond in the main with those obtained in regard to the "masked" iron present. When the warm glycerine and sulphide mixture is applied for about a week, the cytoplasm of young mycelia gives a diffuse reaction for iron, while a deeper one appears in the large granules referred to as affected by hæmatoxylin. In the cytoplasm and granules of the gonidiophores a relatively deeper reaction makes its appearance, and a marked one is obtained in the sterigmata. In the immature gonidia the reaction is diffuse, a special one at the same time obtaining in granules collected or scattered in the cytoplasm. In mature gonidia the granules are larger, and give a deeper reaction for iron, the cytoplasm otherwise showing no trace of its presence (fig. 7). The same results are obtained, but more readily, when sulphuric acid alcohol has been employed to liberate the iron present.

Bacteria.—The question of the occurrence in bacteria of a substance like the chromatin of more highly developed organisms has been investigated to a certain extent by

Ernst,[1] Babes,[2] Wahrlich,[3] Bütschli,[4] Trambusti and Galeotti.[5] Ernst found in a large number of species of bacteria granules which stain with hæmatoxylin and other dyes, while the surrounding protoplasm is coloured faintly or not at all. These, which on account of their direct transformation into spores he termed sporogenous, undergo in their earlier stages solution in artificial gastric juice, but in the more advanced condition resist digestion. From Babes' observations, which agree in the main with those of Ernst, it would appear that the granules which absorb and retain colouring matters and take part in spore formation, also stand in some relation to the division of the bacterial cell. According to Wahrlich, the protoplasm is formed of two constituents at least, a ground substance of reticular structure resembling linin, and one forming granules distributed in this reticulum, and, owing to its capacity for absorbing and retaining dyes, regarded by him as chromatin. In Bacillus pseudoanthracis the small granules which appear before the spores are formed are constituted of chromatin, and from them is derived the main portion of each spore, while the plastin serves apparently for the construction of the spore membrane. Bütschli found in species of Beggiatoa, Chromatium, in Spirochæte serpens, Spirillum undula, Bacterium lineola, and in some Cyanophyceæ, a faintly stainable peripheral portion, and a central body readily stainable, in which a honey-comb structure (Wabenbau) was distinctly

[1] "Ueber den Bacillus xerosis und seine Sporenbildung," 'Zeit. für Hygiene,' vol. iv, p. 25, 1888; also "Ueber Kern- und Sporenbildung in Bacterien," ibid., vol. v, p. 428, 1889.

[2] "Ueber isolirte, färbbare Antheile von Bakterien," ibid., vol. v, p. 173, 1889.

[3] "Bactericlogical Studies." Reprinted from 'Scripta Botanica,' vol. iii, St. Petersburg, 1890–91. I have not seen this work, and the representation of Wahrlich's observations and views is taken from 'Bot. Central.,' vol. xlix, 1892.

[4] 'Ueber den Bau der Bakterien und verwandter Organismen,' Leipzig, 1890.

[5] "Neuer Beitrag zum Studium der inneren Struktur der Bakterien," 'Centralbl. für Bakt. und Parasitenkunde,' vol. xi, p. 717, 1892.

seen. The central body is, in Bütschli's opinion, a nucleus. In or on this organ were observed granules which became red after treatment with hæmatoxylin, and were identified with the granules described by Ernst. Trambusti and Galeotti found in one stage of a very large bacillus isolated from drinking water, that the whole of the protoplasm stained uniformly and deeply with safranin, while in a later stage of the same the stainable substance was converted into granules, disposed at the periphery and arranged in the form of a garland of oval outline. The granules eventually fused to form a homogeneous garland out of which arose from three to four elliptical rings, at first connected by their ends, but afterwards independent of each other, and in this condition became free. These changes the observers regard as analogous to those of mitosis in the cells of more highly specialised organisms.

Schottelius[1] and Ilkewicz[2] have described structures in the bacterial cell which they regard as nuclei, and Sjöbring[3] claims to have found many of the phenomena of mitosis, as it obtains in the cells of higher organisms, exemplified in bacteria. The results of these observers appear to me to have been due to defective methods of tec[illegible].

I find that in Bacillus [illegible]is, B. anthracis, B. megatherium, B. tuberculosis, [illegible] in the root bacillus, there are granules like those described by Ernst and Babes, and which stain with hæmatoxylin, and in B. pseudosubtilis (?), in which there is only one granule to each rodlet, each granule is developed into a spore, the remaining protoplasm at the same time losing all its affinity for colouring matters. The structures observed are the same whether alcohol, corrosive sublimate, or heat has been employed for their fixation.

Ernst found, as already stated, that the granules, except in the later stages, undergo solution in artificial gastric juice.

[1] "Beobachtung Kernartiger Körper im Innern von Spaltpilzen," 'Centralbl. für Bakt. und Parasitenkunde,' vol. iv, 1888, p. 705.

[2] "Ueber die Kerne der Milzbrandsporen," ibid., vol. xv, p. 261, 1894.

[3] "Ueber Kerne und Theilungen bei den Bakterien," ibid., vol. xi, p. 65, 1893.

This would seem to indicate that they are not constituted of typical chromatin.[1] I have endeavoured to determine whether they contain iron in a "masked" form; but the results of my experiments, except in the case of B. megatherium, have not been decided enough to permit a general conclusion on this point. The organisms are very small, and their size would postulate the occurrence of a very small amount of iron in them, and even in the larger spores. When, therefore, a cover-glass preparation of B. megatherium is treated with sulphuric acid alcohol for twenty-four hours, it is not surprising that the subsequent treatment with an acid ferrocyanide solution should give but a very faint blue reaction. When the granules referred to were under observation they manifested themselves by a blue colour slightly deeper than that apparent in the rest of the protoplasm of the organism. In B. subtilis the granules are the only parts of the bacillus which appear to contain iron, the reaction for which is very faint. I have in none of these forms obtained a reaction with the glycerine and sulphide mixture distinct enough to permit certainty of opinion in regard to this. Sulphate of iron, when present in very minute quantities in preparations, appears less distinct than the same amount of iron when revealed by the Prussian blue reaction, and on this account the apparent absence of the sulphide reaction determines nothing. In some preparations of B. pseudosubtilis the largest granules and the spores gave, after treatment with acid alcohols, a blue reaction with the acid ferrocyanide mixture. The root bacillus gave frequently a diffuse and faint blue reaction under the same conditions.

It is obvious that these organisms are too minute to furnish results which would allow the question, whether they contain "masked" iron, and how it is distributed, to be definitely and decisively answered, and I had to employ other forms, of such a size that no difficulty would be experienced in this respect.

[1] Vandevelde ("Studien zum Chemie des Bacillus subtilis," 'Zeit. für Physiol. Chemie,' vol. viii, p. 367, 1884) states that he has isolated nuclein from B. subtilis.

The most readily accessible form was Beggiatoa alba. This organism, as is well known, manifests itself in five different conditions: long threads composed of cells of varying lengths, shorter filaments also formed of cells usually free and motile, spirillum-like elements, comma-shaped, two-celled, swarming bodies, and simple "cocci." Cover-glass preparations of all these forms, fixed first with heat and subsequently with alcohol, were subjected for about two weeks to the action of the glycerine and sulphide mixture at 60° C., while like preparations were treated with sulphuric acid alcohol for about two hours at a temperature of 30° C. The results of both methods agreed. In the long threads the abundance of sulphur granules causes the cytoplasm to have a reticular, or more properly speaking, a vesicular appearance, brought out very prominently when the glycerine and sulphide mixture has dissolved out the sulphur and at the same time given the cytoplasm a greenish colour, developing into a faint blue on treatment with an acid ferrøcyanide mixture. At times the greenish or the blue reaction appears most prominently in some of the nodal points of the "network," but this is doubtless due to the fact that more of the cytoplasm is condensed at such points. The shorter free, motile filaments, which contain, as a rule, very many fewer sulphur granules, have a more homogeneous cytoplasm, and in these the reaction for "masked" iron obtained was a diffuse one. A similar result was obtained in the examples of the spirillum form. In the comma-shaped forms the reaction obtained was, as a rule, slightly deeper, and it frequently appeared most markedly in the central portions of each of the two cells. In some examples a granule in this central mass gave a marked reaction for iron. I did not succeed in determining the relations of the iron in the "cocci."

So far as these results go they correspond with those obtained when cover-glass preparations of Beggiatoa alba are stained with hæmatoxylin, which colours diffusely the cytoplasm in all the forms, but rarely reveals the existence of special chromatin elements. I have been unable to determine, except in a few comma-shaped elements, the occurrence of the denser

central portion described by Bütschli, and I am inclined to regard the structure observed in the exceptional cases as due to shrinkage caused by the method of preparation. In some of the comma-shaped elements the hæmatoxylin stain demonstrates granules like those which were observed to manifest an iron reaction in the glycerine and sulphide preparations. The use of Löffler's solution of methylene blue, followed by that of a saturated solution of bismarck brown, as recommended by Ernst, stains similar granules in the "comma" elements, and in a few of the spirilla only; but it is doubtful if these may be classed with the "sporogenous" granules of other bacteria revealed in the same way. I have not found that there are any granules in the spirilla which contain "masked" iron, although there is the possibility that spirilla, containing granules, were not present in the preparations made with the glycerine and sulphide mixture or with acid alcohol.

The diffusion of the "masked" iron throughout the cytoplasm of Beggiatoa corresponds, on the whole, with what was observed in the other bacteria, but the interpretation of the results in the latter has an element of obscurity in it. It is evident that the iron-holding compound is not, as a rule, localised in granules or in special structures; and although the distribution of this compound, in Beggiatoa alba at least, corresponds with that of the substance which stains with hæmatoxylin and other dyes, it is uncertain whether the two compounds are identical. It is possible that experiments with some of the larger forms, as, e. g., Beggiatoa mirabilis or Crenothrix Kühniana, may result in determining a solution of the question. Unfortunately I had no opportunity of studying the distribution of iron in such large forms.

I did, however, obtain a few preparations of a form which is possibly allied to Crenothrix, and whose size ($2{\cdot}8$—$3{\cdot}2\,\mu \times 6{\cdot}4$—$8\,\mu$) rendered it favourable for such observations as I had an opportunity of making. This organism grew on the surface of some sewage water collected in the fall of 1893, in which also myriads of examples of Euglena viridis throve. It multiplied by fission. Some of them exhibited rounded

ends, while others had an oval shape, but the majority were cylindrical with flat end-surfaces. Several cover-glass preparations of the organisms, fixed by heat and subsequently placed in alcohol, were made, but no cultivations were attempted, since before its value for the purpose of this investigation was ascertained, the original culture fluid had been thrown away.

One of the cover preparations was subjected to the prolonged action of the glycerine and sulphide mixture, but, as sometimes happened in other cases, no result was obtained. The other two were placed in sulphuric acid alcohol for about eight hours at a temperature of about 25° C., and then treated in the usual way with the acid ferrocyanide mixture. One of the preparations was also stained with eosin, and both were, after being washed in water, dehydrated with alcohol and mounted in balsam. Examples of the organism exhibiting the Prussian blue reaction and the eosin stain are represented in fig. 53. The eosin reveals a large central body, sometimes of irregular shape, and always lying free in a cavity in the markedly reticular cytoplasm. The body in question contains no iron, but in other respects resembles the large body present in Saccharomyces Ludwigii. The iron demonstrated appears to be in a granular form distributed in the trabeculæ of the cytoplasm, though sometimes a very large granule, richly supplied with iron, was found adjacent to, or in contact with, the large central body destitute of iron.

As inorganic iron is a constituent of the sheath and other parts in species of Crenothrix and allied forms (C. Kühniana, Leptothrix ochracea and Cladothrix dichotoma), it is possible that all of the iron observed in the form described, and whose relationship to Crenothrix has been suggested, was not derived from a "masked" compound. The amount of inorganic iron must, however, have been very little, for, in the cover preparations subjected to the prolonged action of the glycerine and sulphide mixture, but a few of the forms gave an immediate reaction for iron. The chief difficulty lies in the fact that through the failure of the last-mentioned

method of liberating the "masked" iron in this organism, it is uncertain whether the iron demonstrated after the use of sulphuric acid alcohol had the distribution it obtained in the living organism, or in the cytoplasm before it was treated with acid alcohol. Apart from these matters it seems to me quite certain that the results indicate the presence of iron in a "masked" form in this organism.

Cyanophyceæ.—These organisms, which are generally regarded as closely related to bacteria, offer, on account of their much larger size, fewer and less formidable difficulties to an investigation of the morphological and micro-chemical characters of their cells, and I have, therefore, endeavoured to give a careful attention to the question of the presence of assimilated iron in them. The determination of the relations of the iron compounds in these organisms has entailed also an investigation of the morphology of their cells, and I have, in consequence, obtained a very large number of results, the description of which is beyond the scope of the present paper. These, and a fuller account of the literature of the subject, I propose to detail on a future occasion, and I now deal with the ascertained facts relating to the iron compounds and, in so far as morphological characters are associated with these, with the structure of the cells themselves.

The literature on the subject of the Cyanophyceæ has grown considerably in the last ten years, but as it is only within the last six that improved technical methods have been employed in the investigation of their structure, a short sketch of the more important publications, which have appeared in the latter period, will suffice for present purposes.

Zacharias found that the cell is constituted of a coloured peripheral part, and an uncoloured central portion of a reticulated or granular structure. In the central portion he observed two substances, one exhibiting the characters of a plastin, the other, which he termed the "central substance" (Centralsubstanz), varying in amount in the different cells, and resembling nuclein in its chemical reactions. In the central portion he found granules destitute of nuclein, and related in

many of their characters to the nucleoli of highly developed vegetable organisms.[1] He considers that the central portion differs very greatly from a nucleus, but whether it performs the functions of the latter he is not prepared to say.

Bütschli,[2] whose observations on the structure of bacteria have been already referred to, found one type of structure prevail in both these and the Cyanophyceæ. The cytoplasm is, according to his view, formed of a faintly stainable peripheral zone, and of a denser, deeply stainable central portion which, in the living Cyanophyceæ, is always uncoloured. Both parts are vesiculated. He found that hæmatoxylin colours the cytoplasm blue, while it gives a red stain to granules situated in the central portion, and, in the nodal points of the vesiculated structures, more especially of those of the peripheral zone. These disappear after subjecting the cells to the action of artificial gastric juice, but he nevertheless regards them as chromatin elements, and he looks upon the central portion as a nucleus. Besides these granules, he found in certain Oscillariæ, in the extreme peripheral portions of the cell, and especially adjacent to the transverse cell walls, others which did not stain with hæmatoxylin, but which exhibited a strong affinity for eosin.

Deinega[3] could formulate no conclusion in regard to the presence or absence of a nucleus in these organisms, and also in regard to the nature of the granules, although he is disposed to regard the latter as formed of an isomer of starch. These, of which he found but one species, stain specially with picrocarmine, and dissolve in weak hydrochloric solutions (0·3 per cent.).

Passing over the observations of Zukal,[4] who appears to

[1] "Ueber die Zellen der Cyanophyceen," 'Botanische Zeitung,' 1890, Nos. 1—5.

[2] Op. cit.

[3] "Der gegenwärtige Zustand unserer Kenntnisse über den Zellinhalt der Phycochromaceen," 'Bulletin de la Soc. impér. des Naturalistes de Moscow,' année 1891, p. 431.

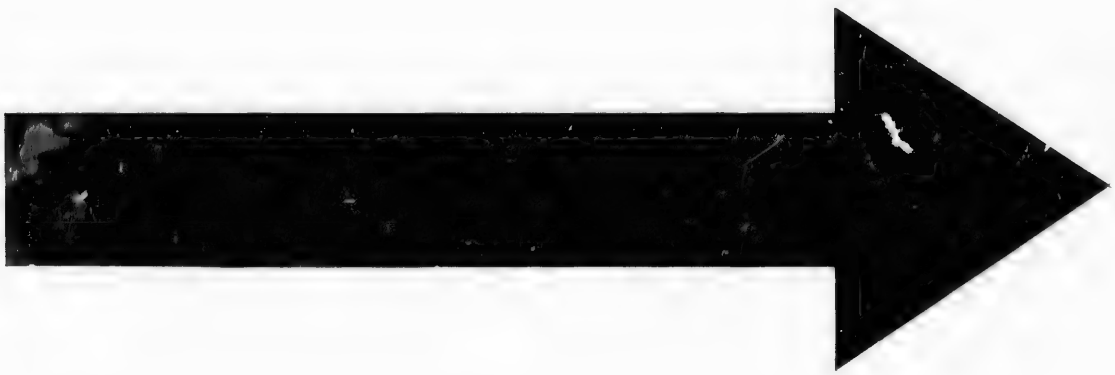
[4] "Ueber den Zellinhalt der Schizophyten," 'Sitzungsber. der K. Acad. der Wiss. Wien,' 1892, Math.-Nat. Classe, vol. ci, p. 301.

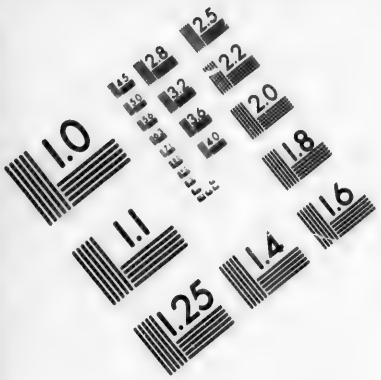
regard all the granules as nuclei, the next investigator of this subject is Hieronymus,[1] who found in these cells a thin hyaline membrane externally, a chromatophore, and a central body consisting of a single much-wound fibril, comprehending in its turns all the granules in the cell. The granules he looks upon as crystals belonging to the regular system, and composed of a substance "cyanophycin," which, though not identical with nuclein in its reactions, he regards as related to the chromatin and pyrenin of highly specialised vegetable cells. The central body is, in his opinion, an "open nucleus."

According to Palla[2] the cells in the Cyanophyceæ consist of a chromatophore with a vesiculated structure, of a central homogeneous body, and of granules of different composition always outside the latter. The central body is affected, like a nucleus, by staining reagents. In preparations fixed with corrosive sublimate and stained with Böhmer's hæmatoxylin the granules adjacent to, or in contact with, the central body are stained reddish-violet, while those scattered in the chromatophore are coloured blue. He finds that those which thus become blue dissolve in dilute solutions of hydrochloric acid (0·3 per cent.) and do not stain intra vitam when treated with solutions of methylene blue. The substance constituting these, and which he calls "cyanophycin," he regards as the first assimilation product of the activity of the chromatophore. Those which stain reddish-violet with hæmatoxylin are composed of a viscid substance, are not soluble in dilute acids, and in the living cell manifest a strong affinity for methylene blue. To such structures he has applied the name "mucous spherules," first given them by Schmitz. They correspond with the granules which, in Bütschli's preparations, stained red with hæmatoxylin, but, in opposition to the views of that observer, Palla regards it as extremely doubtful if they contain any compound comparable to chromatin.

[1] "Beiträge zur Morphologie und Biologie der Algen," Cohn's 'Beiträge zur Biologie der Pflanzen,' vol. v, 1893, p. 461.

[2] "Beitrag zur Kenntniss des Baues des Cyanophyceen-Protoplasts," Pringsheim's 'Jahrbücher für wiss. Bot.,' 1893, vol. xxv, p. 511.





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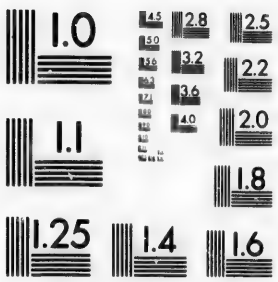

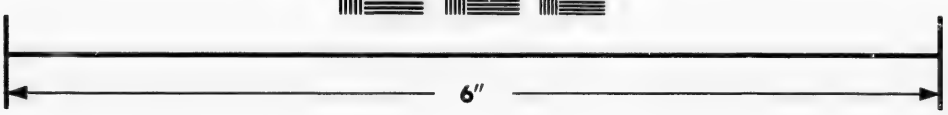

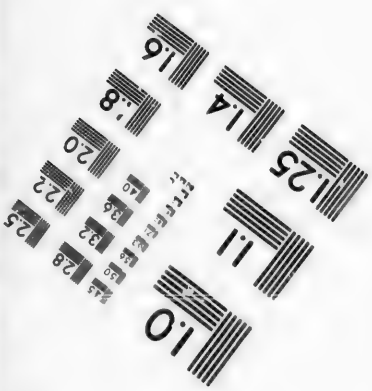

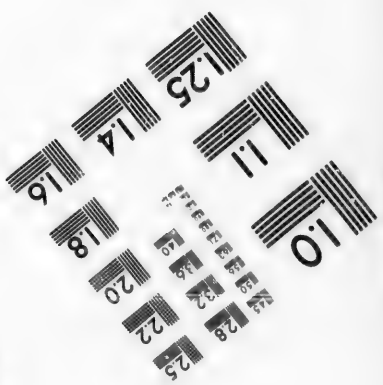

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From all this it may be gathered that nuclei, in the strict sense of the term, are not present in the cells of the Cyanophyceæ, and that if any structure performs the functions of such an organ, it must be the colourless central body. In regard, however, to the composition, the position, and the number of varieties of the granules, there is less of concordance. All the observations quoted would appear to indicate that a typical chromatin substance is absent. If a "masked" iron compound is present in these organisms, with what part of the cell is it associated?

The forms which I used, in endeavouring to determine an answer to this question, were: Oscillaria Froelichii, Oscillaria princeps, Oscillaria sp., Tolypothrix sp., Scytonema sp., Microcoleus terrestris, Cylindrospermum majus, Anabæna (Spherozyga) oscillarioides, and Nostoc commune. The fixative reagents used were alcohol, corrosive sublimate, the stronger Flemming's fluid, and saturated solutions of picric acid; while the staining fluids employed were hæmatoxylin (Ehrlich's and Delafield's), alum cochineal, picro-carmine, safranin, and eosin. In determining the presence of iron compounds, material hardened by alcohol only was used.

The results of my experiments, so far as they affect the question of the relations of iron to the cytoplasm of these cells, may be summarised as follows:

1. The cytoplasm consists of a dense central portion and of a vesiculated peripheral zone, the former staining with hæmatoxylin, alum cochineal, and safranin more deeply than the latter when it is free from granules or vesicles, but when vesicles are present they stain deeply, while the remainder of the central portion acquires a faint colour only slightly more marked than that of the peripheral portion. The size of these vesicles of the central portion varies from that, in which they appear as scarcely larger than granules, to that observed in Tolypothrix sp., in which they measured in diameter a third of that of the cell. The stainable substance of these forms a thick membrane enclosing an apparently inert sub-

stance, and when subjected to the action of artificial gastric juice for two or three days it lessens slightly in volume, but its presence is quite as readily demonstrable then as it was previously. In this case the central portion of the cell also diminishes in volume slightly, the diminution entailing a shrinkage of the peripheral portion away from the original limits of the cell. Digestion does not affect the capacity, on the part of the central substance or of the membrane of the vesicles referred to, of absorbing staining matters, but on subsequently treating such preparations with a solution of potassium hydrate of 0·1 per cent. strength for twenty-four hours, the vesicles disappear and the central body, now somewhat swollen, has lost its capacity for fixing colouring matters in itself. Evidently there is here a substance which has the characters of nuclein. This is confirmed by the results of experiments to determine the presence of "masked" iron. The central body always gives, with the glycerine and sulphide mixture, in an interval of from two or three days to two weeks in length, depending apparently on the size of the cell, a diffuse greenish reaction which is changed to light blue on the addition of a drop of an acid ferrocyanide solution. When granules and vesicles stainable with hæmatoxylin are present, they also give a reaction for iron, but it does not always manifest the same intensity. The iron in them is most readily demonstrated after they have been treated with sulphuric acid alcohol (fig. 51).

2. In the peripheral portions of the cytoplasm, in well-nourished forms only, are granules not so readily stainable with hæmatoxylin, but which are intensely coloured with picrocarmine. These are dissolved out of the fresh cell with dilute hydrochloric acid, and even in preparations thoroughly hardened in alcohol they are but slightly less soluble in the same reagent. In Oscillariæ they are placed in a row at each end of the cell and adjacent to the transverse walls, but in Microcoleus terrestris and Cylindrospermum majus they are disposed in all the peripheral portions of the cytoplasm. In the spores of the latter some of them appear as if embedded

in the central body. These are the "cyanophycin" granules of Palla and such as Bütschli found in Oscillariæ to be unaffected by hæmatoxylin but markedly stained by eosin. They may give a reaction for iron, but not always one of the same intensity, for in Oscillariæ it was very slight, and in one preparation of Microcoleus terrestris none was obtained, while in preparations from the same specimen of fresh material made a few days later than the other, the reaction was quite distinct. In two preparations of Scytonema sp. the granules gave no reaction, a result which I attribute to a deterioration of the solution of the sulphide reagent then used. In Cylindrospermum majus these granules give an intense reaction for iron (fig. 8). The iron is not less firmly combined in the substance of these granules than it is in the chromatin, for in the last mentioned species the glycerine and sulphide mixture brought out its complete reaction only after an application of ten days or more. Within twenty-four hours after the addition of the mixture, they gave, in all the species in which they were iron-holding, a slight greenish reaction. I have not succeeded in demonstrating the presence of iron in them after the use of sulphuric acid alcohol, and the explanation for this is that the latter reagent liberates, but at the same time wholly extracts the iron in these granules, the substance of which, unlike chromatin, is incapable of retaining it.

3. Beyond the fact that the "cyanophycin" granules may contain iron, there is nothing to show a relationship, chemical or physiological, between them and the vesicles. From their situation the "cyanophycin" granules would, as Palla suggested, appear to be the assimilation product of the activity of the chromatophore, while the chromatin vesicles and granules might be regarded as due to processes of elaboration on the part of the central body. In Cylindrospermum majus, which grows on soft mud, the former are usually extremely abundant, but in twenty-four hours after placing the thallus in water, the granules diminish very much in number, and on the third day they may be wholly absent in very many of the filaments. Central vesicles, on the other hand, are in this form extremely

few in number, and the conditions which greatly influence the number of the "cyanophycin" granules have apparently no effect upon them. In Oscillaria Froelichii a filament may contain large numbers of both elements, another may contain "cyanophycin" granules only, while a third may be free from the latter but contain a large number of vesicles, and all in the same preparation. In Cylindrospermum majus the "cyanophycin" granules of the spore diminish somewhat in number and volume during the formation of the episporium, and in the spore which is undergoing its initial division their number is very greatly reduced, the central body appearing at the same time increased in volume.

4. In the heterocysts of Nostoc commune, Cylindrospermum majus, and Scytonema sp., picro-carmine demonstrates the presence of "cyanophycin" substance in a button-shaped body at one or both ends of the cell, according as the heterocyst is terminal or intercalary. A strand of "cyanophycin" connects this body with the contents of the neighbouring cell.[1] In the heterocyst the "cyanophycin" body is quite unconnected with the homogeneous cytoplasm which occupies the remainder of the cavity, and stains but faintly with hæmatoxylin and not at all with picro-carmine. When subjected to the prolonged action of the glycerine and sulphide mixture the "cyanophycin," both of the button and of the strand, gave a deep reaction for iron, and a feebler reaction was obtained in the cytoplasm (fig. 8).

It thus appears that in the Cyanophyceæ there is a substance, containing "masked" iron, in many respects like the chromatin of more highly organized cells, and that the "cyanophycin," a compound of undetermined nature, may, in some forms at least, also give evidence of the presence of the element in a firmly combined condition.

[1] This connection has already been described by Hansgirg, 'Physiologische und Alyologische Studien,' Prague, 1887, pp. 125, 126. The description is quoted in full by Deinega.

General Remarks.

The facts described in the preceding pages appear to indicate that a substance, in which iron is firmly held, is a constant constituent of the nucleus, animal and vegetable, of the cytoplasm of non-nucleated organisms and those possessed of apparently rudimentary nuclei, and that, further, a similar iron-containing substance obtains in the cytoplasm of ferment-forming cells. This substance, to which cytologists apply the term chromatin, cannot, on theoretical grounds, be regarded as constant in its molecular structure, even in the same organism, and its most marked characteristic, apart from the iron in its composition, is the occurrence in it of nuclein or nucleinic acid.

Beyond the fact that the iron is firmly held, it is difficult to say how it is disposed in the molecular structure of the nuclein or nucleinic acid. It is, possibly, united directly to the carbon of the latter. The acid alcohols liberate it as a ferric salt, but this fact cannot be held to indicate that it is combined in the nuclein or nucleinic acid in a ferric state, since from solutions of potassium ferrocyanide, in which the iron is contained in a ferrous state, acids liberate the iron in a ferric condition,[1] as evidenced by the formation of ferric ferrocyanide or Prussian blue.

It is also difficult to say whether there is, in the way in which the iron is held in the animal cell, anything different from that obtaining in the vegetable organism. I have, as a rule, found it easier, in the case of the vegetable cell than in that of the animal cell, to liberate the iron with ammonium hydrogen sulphide; but upon this no conclusion may be founded, since the same reagent liberates the iron of free hæmatin readily, while it does not affect the iron of hæmatin in hæmoglobin, and it is possible that in the animal cell the

[1] The iron immediately on liberation may be in the ferrous state, but it quickly assumes the ferric form. Similarly, the iron liberated in the chromatin may at first be a ferrous compound which, with the continued action of the liberating reagent and under the conditions obtaining in the hardened tissues, may further undergo a conversion into a ferric salt.

proteid molecules attached to the iron-containing nuclein or nucleinic acid may more greatly affect the activity of the reagent than those of the vegetable cell are capable of doing. Since, on the other hand, hæmoglobin, which, as I have pointed out, is derived, in Amblystoma, from chromatin, occurs in a large number of animal forms, but is present in no vegetable organism, it would appear to follow that the iron is combined in animal chromatin in a way unlike that in which it is held in the vegetable cell.[1]

The apparently universal occurrence of such iron compounds renders intelligible the fate of the iron salts absorbed by plants from the soil, and of the iron compounds found by Raulin[2] and Molisch[3] to be necessary for the growth of Aspergillus niger. Chromatin, to the formation of which the iron absorbed contributes, is, as the results of cytological investigations show, a substance of primary importance to the cell, and a diminution in, or a cessation of, the supply of iron to the vegetable organism, which produces the condition known as chlorosis, instead of affecting only the formation of its chlorophyll, as generally supposed, strikes at its very life.

The conditions known as anæmia and chlorosis in the higher Vertebrates have been hitherto explained as caused by a diminished production of hæmoglobin directly from organic or inorganic iron compounds absorbed by the intestine from the food matters; but they must now be referred to a deficient supply of the primary iron-containing com-

[1] Compounds which appear to resemble, somewhat remotely, the hæmatins of animal organisms have been found in Palmella cruenta (Phipson, "Sur la matière colorante du Palmella cruenta," 'Comptes Rendus,' vol. lxxxix, p. 316, 1879), and in Aspergillus niger (Linossier, "Sur une hématine végétale; l'aspergilline, pigment des spores de l'Aspergillus niger," 'Comptes Rendus,' vol. cxii, p. 489, 1891). The colouring matter of the latter is, as I have found, held in the membrane, but not in cryptoplasm of the spore, and it would, therefore, appear to be simply a degeneration product.

[2] "Études chimiques sur la végétation," 'Annales des Sc. Nat.,' Bot., Série 5, vol. xi, 1869, p. 93.

[3] Op. cit., pp. 97—117.

pound, chromatin, not only in the hæmatoblasts, but in all the cells of the body. The consequently lessened proliferation of cell and tissue would explain the hypoplasia of the imperfectly developed vascular system observed by Virchow[1] in chlorotic human subjects.

Accepting this explanation of the nature of chlorosis, one may infer that this condition is not limited to animal organisms in which hæmoglobin is found, although its occurrence in others may be difficult to detect because of the total absence of this pigment. From this point of view animal chlorosis is fundamentally similar to the chlorosis of the vegetable kingdom.

The oxygen-carrying property of hæmoglobin and of hæmatin is generally attributed to the iron present in these, because when hæmatin is deprived of its iron, the resulting compound, whether hæmatoporphyrin or bilirubin, manifests no affinity for oxygen. The proof may not be quite conclusive, for we cannot be certain that either compound represents the unchanged remainder of the hæmatin less its iron, but assuming that it is correct, it follows, as I have pointed out in my previous communication, that the antecedent of hæmoglobin, chromatin, has the capacity of absorbing and retaining oxygen, and that one may attribute the processes grouped under the term "vital," to an alternation of the conditions of oxidation and reduction in the iron-holding nuclear constituent. This hypothesis, reasonable as it now appears to me to be, I do not regard as free from difficulties, since in vegetable cells the two processes of respiration and assimilation, involving two activities of different natures, so far as the oxygen is concerned, appear to postulate the existence of two different iron compounds in the same nucleus.[2] There are no facts to indicate

[1] "Ueber die Chlorose und die damit zusammenhängenden Anomalien im Gefässapparate, insbesondere über Endocarditis puerperalis," 'Vortrag.,' Berlin, 1872.

[2] On the relations of the vegetable nucleus to the processes of assimilation, see Strasburger, 'Ueber Kern- und Zelltheilung im Pflanzenreiche,' 1888, pp. 194—204.

the occurrence of such, and it is scarcely possible to explain away the objection without advancing some hypotheses regarding the action of the sulphur and the phosphorus in the nuclein. I propose to detail these on another occasion.

EXPLANATION OF PLATES 10—12,

Illustrating Dr. A. B. Macallum's paper "On the Distribution of Assimilated Iron Compounds, other than Hæmoglobin and Hæmatins, in Animal and Vegetable Cells."

Explanation of Figures.

Note.—In the preparation of all the figures Abbé's camera lucida was employed when the size of the objects represented permitted its use, and all except 25, 26, 35, and 36 are illustrated as they were seen with an apochromatic immersion objective (Zeiss 3 mm., 2 mm., or 1·5 mm.). The exceptions are represented as they appeared under a Zeiss D. Figs. 1—40 show the distribution of assimilated iron as it was demonstrated by the dark green colour of ferrous sulphide, but in Figs. 41—53 the disposition of iron compounds of this kind is indicated by the colour of the Prussian blue reaction.

Fig. 1.—A nucleus and a cell from the testicle of Necturus lateralis. Alcohol, the glycerine and sulphide mixture eleven days. × 620. This and the two succeeding illustrations were drawn from the very first preparations made with this reagent.

Fig. 2.—Testicular elements of another example of N. lateralis. Alcohol, the glycerine and sulphide mixture eleven days. × 620.

Fig. 3.—*a*, a leucocyte, *b*, a red corpuscle, of N. lateralis. Alcohol, the glycerine and sulphide mixture six days. × 500.

Fig. 4.—Two yeast-cells, Saccharomyces cerevisiæ. Alcohol, the glycerine and sulphide mixture ten days. × 1500.

Fig. 5.—Four yeast-cells, Saccharomyces Ludwigii. Alcohol, the glycerine and sulphide mixture four days. × 1640.

Fig. 6.—The developing and fully-formed spores of Cystopus candidus. *a*, *b*, *c*, *d*, *e*, alcohol, sulphuric acid alcohol two days, ammonium hydrogen sulphide in glycerine. × 750. *f*, alcohol, the glycerine and sulphide mixture ten days. × 680.

FIG. 7.—Spores of Aspergillus glaucus, *a*, in the unripe, *b*, in the ripe condition. Alcohol, the glycerine and sulphide mixture three days. × 1640.

FIG. 8.—Cells, heterocyst (*h.*), and spore (*sp.*) of Cylindrospermum majus. Alcohol, the glycerine and sulphide mixture fourteen days. × 1640.

FIG. 9.—Three cells of a filament of Microcoleus terrestris. Alcohol, the glycerine and sulphide mixture four days. × 2000.

FIG. 10.—*a*. Spores (immature), *b*, *c*, and *d*, basidia of a leucosporous Hymenomycete. *d*. Basidium with sterigmata and one attached spore. Sterile (?) element in *b*. Alcohol, the glycerine and sulphide mixture eight days. × 820.

FIGS. 11—13.—Portions of hyphæ of Hyphelia terrestris Fries, illustrating the development of the fructification, 13 *a* and *b* representing the simplest form. Alcohol, the glycerine and sulphide mixture three days. × 820.

FIGS. 14—18.—From the ovary of a specimen of Erythronium americanum hardened in alcohol. Fig. 14 illustrates the effect produced by diammonium sulphide and glycerine in two days; Figs. 15, 17, and 18 represent that produced by ammonium hydrogen sulphide and glycerine in the same time; and in Fig. 16 is shown how intense the reaction appeared after treatment for four days with the same reagent. × 1240.

FIGS. 19—22.—From the ovary of a specimen of Erythronium americanum hardened in alcohol. Sections treated for thirty hours with sulphuric acid alcohol, and mounted in a mixture of glycerine and ammonium hydrogen sulphide. × 1240.

FIG. 23.—Four hepatic cells from a specimen of Necturus lateralis. Alcohol, the glycerine and sulphide mixture eight days. × 620.

FIG. 24.—Two hepatic cells from the same animal, illustrating the distribution of the iron and the nuclear structure after they were treated with sulphuric acid alcohol for twenty-four hours, and mounted in a mixture of glycerine and ammonium hydrogen sulphide. × 620.

FIG. 25.—An example of Stentor polymorphus. Alcohol, the glycerine and sulphide mixture two weeks. × 305.

FIG. 26.—An example of Stentor polymorphus. Alcohol, Bunge's fluid thirty-seven hours, ammonium hydrogen sulphide and glycerine. × 305.

FIG. 27.—Examples of Vorticella sp. Alcohol, the glycerine and sulphide mixture seven days. × 600.

FIG. 28.—An example of Epistylis sp. Alcohol, Bunge's fluid twenty-four hours, glycerine and ammonium hydrogen sulphide. × 600.

FIG. 29.—An ovum of Ascaris mystax, fixed during impregnation. Only a portion of the ovum is represented. Alcohol, the glycerine and sulphide mixture eight days. × 820.

FIG. 30.—An impregnated ovum of Ascaris mystax, showing the division of its nucleus (*n.*) and the condition of the spermatozoid (*sp.*). Alcohol, the glycerine and sulphide mixture ten days. × 750.

FIGS. 31 and 32.—Spermatozoids of Ascaris mystax. Alcohol, the glycerine and sulphide mixture nine days. 31, × 820; 32, × 1640.

FIGS. 33—36.—Ovarian ova of the lake-lizard, Necturus lateralis, illustrating differences in the distribution of the "masked" iron. In 5 is shown the iron-containing peripheral nucleoli, and *a* represents a more highly magnified (= × 1240) portion of the nuclear structure. In 36 is seen an earlier stage with *a*, a portion of its nuclear network, more highly magnified (× 1240). Alcohol, sulphuric acid alcohol thirty-six hours, glycerine and ammonium hydrogen sulphide. × 305.

FIG. 37.—Retinal rods and cones from a larva of Amblystoma. Alcohol, whole of retina in Bunge's fluid two days, glycerine and ammonium hydrogen sulphide. × 620.

FIG. 38.—Cells from the pancreas of a larva of Amblystoma. Alcohol, Bunge's fluid (on the whole of the organ) two days, glycerine and ammonium hydrogen sulphide. × 620.

FIG. 39.—A portion of a section of the human epidermis, illustrating the occurrence of "masked" (?) iron in the granules (eleidin) of the stratum granulosum and in the stratum lucidum. Alcohol, sulphuric acid alcohol two days, glycerine and ammonium hydrogen sulphide.

FIG. 40.—Strands of fibrils from the muscle of a larva of Amblystoma. Alcohol, sulphuric acid alcohol two days, glycerine and ammonium hydrogen sulphide. × 750.

FIGS. 41 *a* and *b*.—From the ovary of a specimen of Menopterium americanum; *b* represents an isolated nucleus. Alcohol, sulphuric acid alcohol thirty hours, acid ferrocyanide mixture, balsam. × 1240.

FIG. 42.—A cell from a section of the ovary of the same specimen, with the iron demonstrated as in last case, but the preparation, before being mounted in balsam, was stained with eosin. × 1240.

FIGS. 43 and 44 *a* and *b*.—Nuclei of the embryo sac of a specimen of E. americanum. Alcohol, sulphuric acid alcohol thirty-six hours, acid ferrocyanide mixture, balsam. × 620.

FIGS. 45 *a* and *b*.—Nuclei from the liver of a specimen of Necturus lateralis. *n.* Nucleoli. Alcohol, sulphuric acid alcohol thirty-six hours, acid ferrocyanide mixture, balsam. × 1240.

FIGS. 46 *a*—*d*.—Hepatic nuclei treated as in foregoing case, also stained with safranin to illustrate the differences between the chromatin network and the nucleoli in regard to the effect of this reagent. × 1240.

FIG. 47.—Two hepatic nuclei treated as in the preparation illustrated by

Fig. 45, but also stained with eosin, which deeply colours the nucleoli and non-iron-containing constituents (see text). × 1640.

Fig. 48.—Nuclei of the epithelial cells of the intestinal mucosa of Necturus lateralis. Alcohol, sulphuric acid alcohol thirty-six hours, acid ferrocyanide mixture, balsam. The preparation from which *b* was drawn was, before being mounted in balsam, stained with safranin. × 1240.

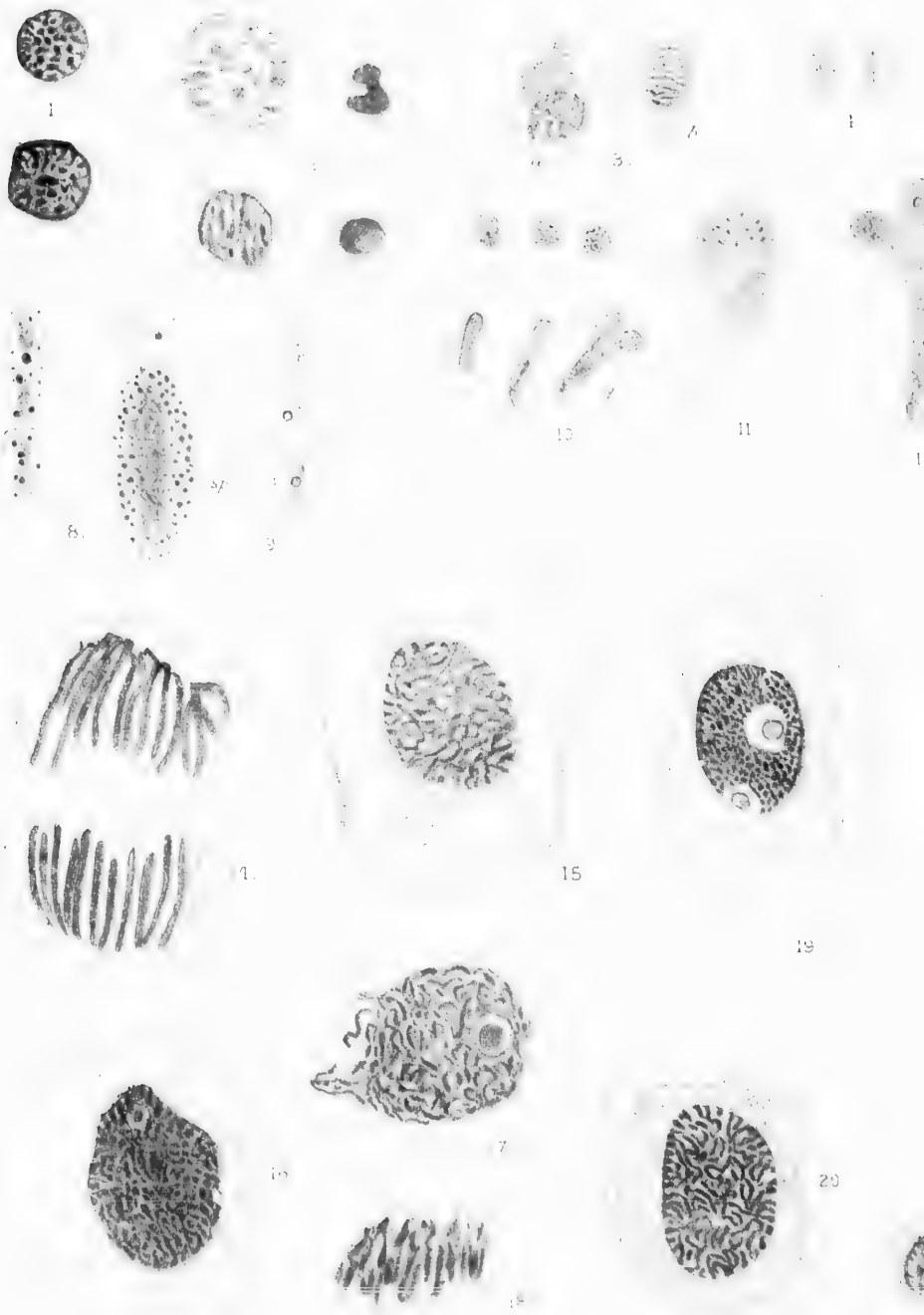
Figs. 49 *a* and *b*.—Two examples of Euglena viridis. Alcohol, sulphuric acid alcohol thirty-six hours, acid ferrocyanide mixture, balsam. × 820.

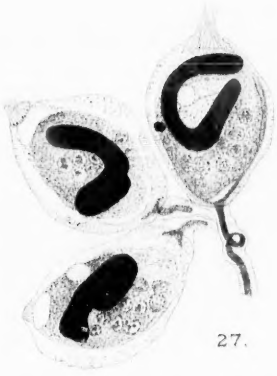
Fig. 50.—A portion of a nuclear filament from the "salivary" gland of a larva of Chironomus. Alcohol, sulphuric acid alcohol thirty-six hours, acid ferrocyanide mixture, balsam. × 1640.

Figs. 51 *a*, *b*, *c*, *d*.—Cells and portions of filaments of Oscillaria Froelichii; *b* and *d* represent the isolated cells as seen through their transverse walls. The "cyanophycin" granules are coloured red. Alcohol, sulphuric acid alcohol three hours, acid ferrocyanide mixture, picro-carmine, balsam. × 1640.

Fig. 52.—A portion of a filament of Microcoleus terrestris. Alcohol, sulphuric acid alcohol three hours, acid ferrocyanide mixture, balsam. × 1500.

Fig. 53.—Examples of an organism obtained from sewage water (see text). Alcohol, sulphuric acid alcohol eight hours, acid ferrocyanide mixture, balsam. × 2000.





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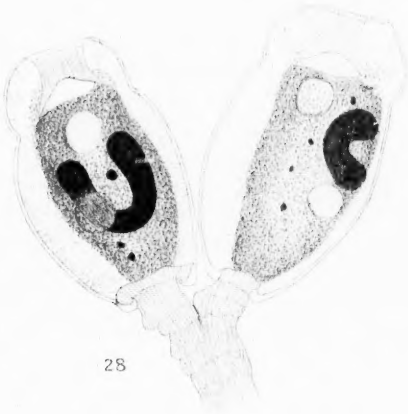

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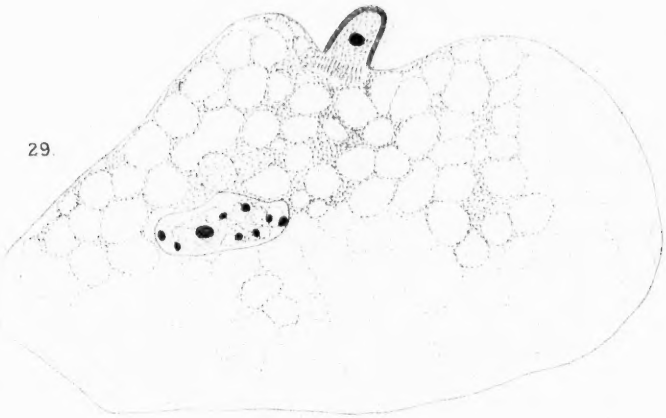

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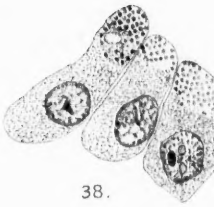

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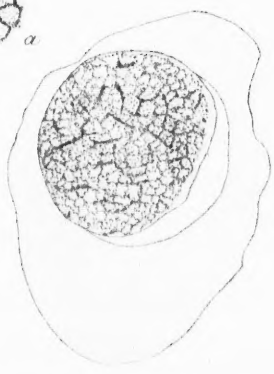

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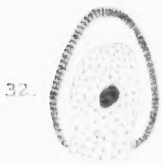

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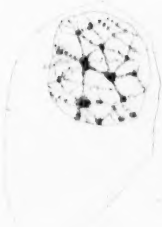

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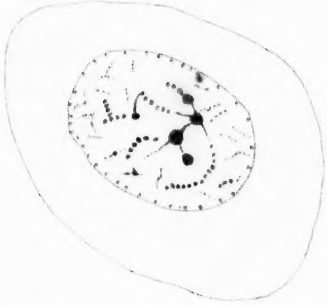

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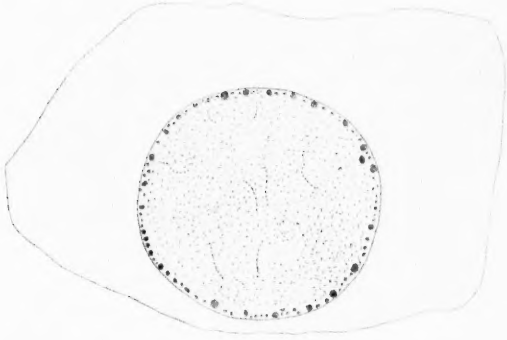

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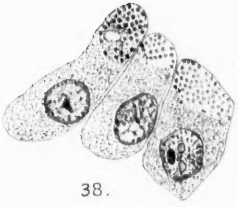

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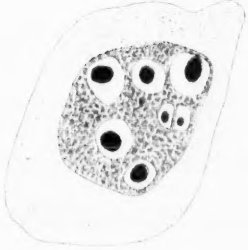

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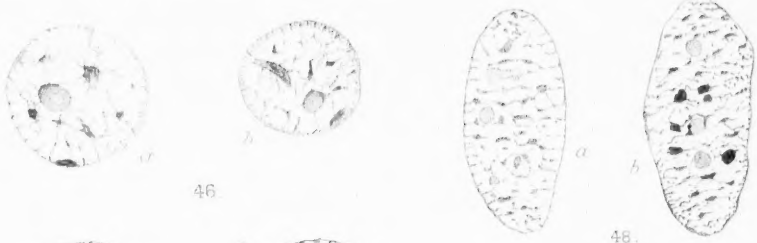
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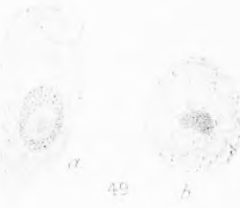
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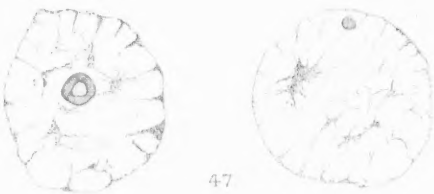
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